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### CORRECTION

On pages 737, 740, 742, 744, 763, and 765, Vol. 117, No. 2, February, 1937, the foot-note to the tables should read: To show a significant difference between the mean number of lesions in any one experiment, the ratio of the mean difference (M.D.) to the standard error of the mean difference (S.D.) should not be less than 2.1.



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## SYNTHESIS OF $\alpha$ -AMINO- $\beta$ -HYDROXY-*n*-BUTYRIC ACIDS. II

By MARION L. WOOD, ROBERT J. MADDEN, AND  
HERBERT E. CARTER

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(Received for publication, September 21, 1936)

The purpose of the present paper is to summarize briefly the detailed study which has been made of the method used by Abderhalden and Heyns (1) in synthesizing  $\alpha$ -amino- $\beta$ -hydroxy-*n*-butyric acid. This study was undertaken for several reasons. In the first place we were unable to duplicate exactly Abderhalden's results. Furthermore, it was discovered that, when the synthesis was carried through with no purification of the intermediates, the final product exhibited slight growth-promoting activity. It seemed possible, therefore, that by varying the conditions a mixture might be prepared containing a high per cent of the active form. While the work was in progress, Mayeda (2) reported the synthesis, by Abderhalden's method, of amino-hydroxybutyric acid which was said to support growth in 1 per cent. If this observation is correct, the product must have been mainly, if not entirely, *dl*-threonine, assuming that only one enantiomorph of *dl*-threonine is physiologically active, as seems likely. The fact (3) that  $\alpha$ -amino- $\beta$ -hydroxy-*n*-butyric acid prepared from purified intermediates and yielding the derivatives reported by Abderhalden was physiologically inactive casts some doubt on the accuracy of Mayeda's results. However, it is not impossible that he might have employed conditions yielding mainly *dl*-threonine and this possibility necessitated an even more thorough study of the reactions.

The results obtained will be summarized briefly. Unless otherwise specified the conditions employed were those used by Abderhalden. In order to distinguish the two forms of the various intermediate compounds the suffix A will be used to indicate the

substances related to the aminohydroxybutyric acid prepared by Abderhalden's method and the suffix B to indicate the precursors of the second or growth-promoting form.

*Ethyl  $\alpha$ -Bromomercuri- $\beta$ -Methoxy-*n*-Butyrate*—In the preparation of this compound variations were made in the time and temperature of mercuration and in the temperature at which the aqueous potassium bromide was added. Mercuric acetate was replaced with equivalent quantities of mercuric oxide and glacial acetic acid. Potassium bromide was replaced with hydrobromic acid, since Mayeda indicated in equations that the latter reagent was used. In every case the product obtained melted sharply at 86.5–87° after recrystallization from ethyl or methyl alcohol, or a mixture of ethyl acetate and petroleum ether. The range of yields was excellent except when hydrobromic acid was used in forming the bromomercuri ester. In view of the well known instability of mercuric acetate addition compounds toward strong acids, this result is not surprising.

Abderhalden reported a melting point of 78–79° for ethyl  $\alpha$ -bromomercuri- $\beta$ -methoxy-*n*-butyrate. Either his product was slightly impure or we had prepared the second form. The former view seemed more plausible since our bromomercuri ester yielded the intermediate and final products reported by Abderhalden. It was hoped to settle this point by the preparation of the second form from ethyl isocrotonate, but the heavy oil obtained has not crystallized as yet.

*Ethyl  $\alpha$ -Bromo- $\beta$ -Methoxy-*n*-Butyrate*—The brominations were carried out in chloroform and ethyl acetate as solvents, in the presence and absence of sunlight, and with crude and purified bromomercuri ester. This was the only step in which epimerization was detected. The nature of the product varied widely with different conditions. Attempts to separate the mixtures by fractionation were only partially successful. The boiling point range was so slight that the fractionation was followed by refractive indices. From two to four refractionations yielded pure ethyl  $\alpha$ -bromo- $\beta$ -methoxy-*n*-butyrate-A, whose refractive index was 1.4500. This ester was the largest constituent of the mixtures, particularly when the brominations were carried out in the absence of sunlight. There was obtained also a series of slightly higher boiling fractions whose refractive indices ranged from

1.4530 to 1.4875. No pure substance was isolated from these, although there is little doubt that they contained mainly ethyl  $\alpha,\beta$ -dibromo-*n*-butyrate, since the saponification equivalents of the last fractions were close to the theoretical value and both  $\alpha$ -bromocrotonic and  $\alpha$ -bromoisocrotonic acids were obtained from them on hydrolysis.

Since ethyl  $\alpha$ -bromo- $\beta$ -methoxy-*n*-butyrate-B could not be isolated by fractionation, the relative amounts of the two forms present in a mixture was roughly ascertained by carrying a sample through to the aminohydroxy acid and determining the physiological activity of the latter. In this way it was found that sunlight was the most important factor influencing the nature of the product. In the absence of sunlight the bromination proceeded slowly and irregularly in the cold. When the reaction mixture was warmed to 40–50°, the utilization of the bromine proceeded smoothly and rapidly after an initial lag period. Heat was evolved and the temperature was held between 40–50° by a water bath. The products contained mainly ethyl  $\alpha$ -bromo- $\beta$ -methoxy-*n*-butyrate-A with small amounts of the dibromo ester. The aminohydroxy acid mixtures obtained from these products were only slightly active physiologically. Apparently, in the absence of sunlight the reaction occurred without appreciable rearrangement, yielding only the physiologically inactive form. When the brominations were carried out in the presence of sunlight, the reaction proceeded much more rapidly and cooling in an ice bath was necessary. The product contained considerable amounts of ethyl  $\alpha,\beta$ -dibromo-*n*-butyrate and a much higher per cent of ethyl  $\alpha$ -bromo- $\beta$ -methoxy-*n*-butyrate-B. Different samples yielded aminohydroxy acid mixtures which supported growth in 2.5 to 4 per cent. The majority were active in 2.5 to 3 per cent.

*$\alpha$ -Bromo- $\beta$ -Methoxy-*n*-Butyric Acid*—Although Abderhalden stated that 2 to 3 hours were required to hydrolyze the bromo ester, we found that 10 minutes vigorous shaking with 0.5 N sodium hydroxide is sufficient. Increasing the time of shaking to 4 hours slightly decreases the yield but does not affect the nature of the acid mixture obtained. In all subsequent work the esters were hydrolyzed by shaking them for 10 minutes with 0.5 N sodium hydroxide.

Hydrolysis of ethyl  $\alpha$ -bromo- $\beta$ -methoxy-*n*-butyrate-A and of crude products from the bromination in the absence of sunlight gave a viscous oil which partially solidified on long standing in a vacuum desiccator over phosphorus pentoxide. The solid material was separated from the oil by suction filtration. The solid was crude  $\alpha$ -bromo- $\beta$ -methoxy-*n*-butyric acid-A. After several recrystallizations from a benzene-petroleum ether mixture the bromo acid-A melts at 62-63°. Abderhalden reported a melting point of 59-60°.

The bromo esters obtained on bromination in the presence of sunlight gave on hydrolysis no crystalline product other than  $\alpha$ -bromocrotonic and  $\alpha$ -bromoisocrotonic acids.  $\alpha$ -Bromo- $\beta$ -methoxy-*n*-butyric acid-B has not been isolated in the crystalline state.

The bromo acids distil at 105-106°, at 2 mm., without undergoing appreciable epimerization.

*$\alpha$ -Amino- $\beta$ -Hydroxy-*n*-Butyric Acid*—In converting the bromo acids to aminohydroxy acids the time and temperature of amination and the time of refluxing with 48 per cent hydrobromic acid were varied with no effect on the nature of the product. It was found, however, that extending the time of amination to 5 hours gave slightly better yields.

*Preparation of  $\alpha$ -Amino- $\beta$ -Hydroxy-*n*-Butyric Acid-A*—The crude crystalline bromo acid-A was aminated for 5 hours and the amination mixture was worked up in the usual manner. It is much better to employ the crystalline bromo acid as the starting material. If the bromo acid used contains a small amount of bromo acid-B, it is difficult to obtain pure  $\alpha$ -amino- $\beta$ -hydroxy-*n*-butyric acid-A by fractional crystallization of the mixture obtained.

*Preparation of Mixture of  $\alpha$ -Amino- $\beta$ -Hydroxy-*n*-Butyric Acid-A and B*—Crude bromomercuri ester was dried thoroughly to remove methyl alcohol and water. 425 gm. of the dry ester were dissolved in 1 liter of chloroform and the solution was placed in a 3 liter flask exposed to direct sunlight. 160 gm. of bromine were added as it was utilized. The reaction was rapid, requiring about 30 minutes. Considerable heat was evolved and better yields were obtained if the flask was cooled in an ice bath during the addition of the bromine. The crude bromo ester produced was carried through to the aminohydroxy acid in the usual manner. The mixture

of aminohydroxybutyric acids obtained supported growth of rats when included in their diets in 2.5 to 4 per cent.

#### SUMMARY

A direct method is now available for preparing a mixture of aminohydroxybutyric acids which supports growth of rats when included in their diets in 2.5 to 4 per cent. This is a considerable improvement in yield and time required over the synthetic method formerly used (3).

It is difficult to understand the results reported by Mayeda. In the absence of any data or comment concerning his product it seems logical to assume that Mayeda duplicated the results of Abderhalden, in which case the amino acid obtained would have been physiologically inactive. The other possibility, that Mayeda prepared nearly pure *dl*-threonine and failed to characterize it by physical properties or derivatives, seems improbable in view of our failure to obtain a product by this method which supported growth of rats when included in their diets in less than 2.5 per cent.

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## CHEMICAL STUDIES OF CALLICREIN

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(Received for publication, September 21, 1936)

A comparison of the methods used for the separation and purification of callicrein with those used for prolan reveals a similarity in the physical properties shown by these two substances. In the crude fractions they follow the protein precipitates; more highly purified, they are not precipitated by the protein precipitants. They are 50 per cent alcohol-soluble and non-dialyzable. Both are readily adsorbed by the acid-insoluble, naturally occurring urine colloids and other adsorbing agents. Highly purified, they are precipitated from 80 per cent alcohol only in the presence of electrolytes. The methods used for the purification of these substances are based on the properties mentioned above and there is no procedure of purification yet described which would indicate a complete separation of one substance from the other. Callicrein differs from prolan in that it is rapidly destroyed at a pH more acid than 4.0, and is highly unstable when purified to 1000 units per mg. The crude callicrein preparations are stable in aqueous solution (pH 4.5 to 8.5) indefinitely, whereas prolan gradually loses its activity in aqueous solution.

It is therefore quite likely that even highly purified prolan preparations are contaminated with inactivated callicrein.

It was therefore of interest to study the behavior of callicrein when subjected to procedures similar to those used in the chemical studies of prolan (1).

### EXPERIMENTAL

The callicrein used in these studies was prepared and assayed by the methods previously reported (2) and represented a stable product as pure as any described. Since the reagents themselves,



when given intravenously, might produce vascular changes, a blank assay of the reagents in the concentrations used was made simultaneously with that of the reaction mixture. It will be noted that benzoyl chloride was the only reagent to give an appreciable blank. The assays were not carried beyond the point at which 90 per cent inactivation was assured.

The reagents, their concentration, the reaction volume, time of reaction, and the respective assays are given in Table I. The reactions took place at room temperature.

TABLE I  
*Influence of Reagents upon Activity of Callicrein*

| Reagent added<br>(room temperature) | Reaction time | Assay after<br>reaction | Assay for<br>blank effect<br>of reagents | Per cent<br>recovery |
|-------------------------------------|---------------|-------------------------|--|----------------------|
|                                     | <i>hrs.</i>   | <i>units per cc.</i>    | <i>units per cc.</i>                     |                      |
| None (Control)                      | 24            | 720                     | <20                                      | 100                  |
| 30 mg. $\text{Me}_2\text{SO}_4$     | 24            | 95                      | <65                                      | 15                   |
| 15 " "                              | 24            | 13                      | <10                                      | 2                    |
| 30 " $\text{Ac}_2\text{O}$          | 24            | 40                      | <20                                      | 6                    |
| 15 " "                              | 24            | 72                      | 72                                       | <10                  |
| 60 " $\text{BzCl}$                  | 24*           | 163                     | 163                                      | <25                  |
| 50 " $\text{CS}_2$                  | 1             | 720                     | <65                                      | 100                  |
| 50 " "                              | 24            | 225                     | <20                                      | 30                   |
| 20 " $\text{HCHO}$                  | 1             | 520                     | <65                                      | 70                   |
| 20 " "                              | 2             | 60                      | <65                                      | 8                    |
| Control at 98°                      | $\frac{1}{2}$ | <20                     | <20                                      | <3                   |
| 20 mg. $\text{PhCNO}$               | 24*           | 175                     | <20                                      | 25                   |
| 50 " $\text{AcH}$                   | 24            | 720                     | <10                                      | 100                  |

In each experiment the reaction mixture contained 6 mg. of callicrein and 100 mg. of  $\text{NaHCO}_3$  in a 5 cc. volume.

\* Insoluble reaction products removed by filtration.

### *Results*

The results show that highly purified callicrein is 90 per cent inactivated by methylation and acylation and largely inactivated by the condensation reagents,  $\text{CS}_2$  and phenyl isocyanate. It is stable to acetaldehyde but not to formaldehyde. In studies on a cruder preparation (3), we had previously reported inactivation by iodine and peroxide and stability to formaldehyde. In the present studies, the formaldehyde concentration was 10 times that used in the earlier studies. It will be noted that inactivation by formaldehyde is not instantaneous.

Of the reagents studied, those which inactivated prolan also inactivated callicrein: water at 98°, acylation, reaction with  $\text{CS}_2$ , phenyl isocyanate, iodine, peroxide. Callicrein is more sensitive to acidity than prolan. Both are stable to mild reducing agents, being inactivated by formaldehyde only when present in excess. Callicrein is definitely more sensitive to methylation than prolan. In fact, the relative stability of prolan to methylation, in contrast to its instability to other reagents, must be regarded as characteristic.

*Physiologic Significance of Callicrein*—The biologic standardization of callicrein is performed by measuring the effect of an intravenous injection. There can be no question about the accuracy or sensitivity of this test. In this laboratory, however, we have consistently failed to note any circulatory response produced by callicrein when administered either intramuscularly or intraperitoneally in doses as high as 5000 units per kilo. On the basis of this negative response, less than 1/5000 of the dose given intramuscularly or intraperitoneally could appear at any time in the blood stream as callicrein. Callicrein produces a fall in blood pressure when given intravenously. Adrenalin should be liberated in this process as a defense mechanism and a rise in blood sugar should occur. In four rabbits given 500 to 1000 units of callicrein per kilo intravenously blood sugar increases of 64, 36, 14, and 30 mg. per 100 cc. were noted 1 to 3 hours after dosage. 5000 units per kilo given intramuscularly, however, produced no rise in blood sugar. The subcutaneous administration of callicrein in the treatment of hypertension, prompted by the mistaken idea that vascular changes occur similar to those resulting from intravenous administration, is therefore hardly justified. It is above all pertinent to find out why intravenous administration alone produces a physiologic effect. No further chemical studies on callicrein in this laboratory are contemplated until the latter question is answered.

#### SUMMARY

The conditions under which a highly purified stable fraction of the depressor colloid of urine (callicrein) is inactivated by methylation, acylation, reaction with  $\text{CS}_2$ , phenyl isocyanate, and formaldehyde are described. This depressor colloid, which resembles

the prolan fraction in physical properties, is if anything less stable to chemical reagents than prolan.

Since the physiologic effects produced by an intravenous injection of callicrein cannot be duplicated by even massive doses when given intramuscularly or intraperitoneally, the physiologic significance of callicrein is questionable.

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# FURTHER OBSERVATIONS ON THE ASSAY, DISTRIBUTION, AND PROPERTIES OF THE FILTRATE FACTOR

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In a previous communication (1) data were presented concerning the distribution of the "filtrate factor," a water-soluble vitamin belonging to the vitamin B complex and preventing a dietary dermatitis in chicks (vitamin B<sub>2</sub> of Elvehjem and Koehn (2)), in certain feedingstuffs. The present communication reports further assays of feedingstuffs for the factor, and a unit is proposed for the biological assay of the factor. The name "filtrate factor" is not proposed as a permanent title for the vitamin, but is used, as previously (1,3), for the sake of brevity and specificity

## EXPERIMENTAL

Details of the care of chicks and the production of the syndrome have been described previously (1, 3, 4). The basal heated diet, first described by Kline, Keenan, Elvehjem, and Hart (5), was modified until it now has the following composition (Diet 80-G).<sup>1</sup>

|   |      |                                 |
|---|------|---------------------------------|
| Yellow corn-meal.....   | 55.5 | } Heated to 120°<br>for 36 hrs. |
| Wheat middlings.....  | 25   |                                 |
| Commercial casein.....  | 12   |                                 |
| Sodium chloride.....  | 1    |                                 |
| Ground limestone.....   | 1    |                                 |
| Steamed bone-meal.....  | 1    |                                 |
| Cod liver oil, medicinal.....   | 2    |                                 |
| Whey adsorbate.....   | 2.5  |                                 |
| Hexane extract of alfalfa meal corresponding to 1% alfalfa meal,<br>evaporated on the diet. |      |                                 |

<sup>1</sup> Casein was generously furnished by the California Milk Products Company, Gustine, California, by the cooperation of Mr. J. Chrisman.

Whey adsorbate<sup>3</sup> was prepared by stirring 1 kilo of English fullers' earth with about 150 liters of commercial hydrochloric acid whey at pH about 6.5 for 30 minutes. The fullers' earth was removed by filtration, washed, and dried. The whey adsorbate was a potent source of lactoflavin. 1 gm. of whey adsorbate furnished about 50 modified Bourquin and Sherman units of vitamin G (flavin) when assayed by the method of Dimick, Smith, and Davis

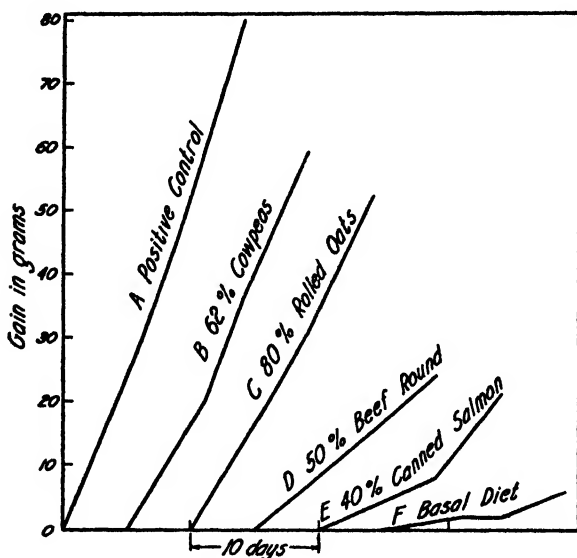


FIG. 1. Growth of chicks on heated diets supplemented with various feedingstuffs. Ten chicks were used in each group. A preliminary depletion period of 6 days on the basal diet was followed by an assay period of 13 days on the test diets. The syndrome score (4) at the end of the assay period was as follows: for the group represented by Curve A, 0; Curve B, 0; Curve C, 0; Curve D, 16; Curve E, 17; Curve F, 14. The diets are described in Table I and in the text.

(6). 2.5 per cent of whey adsorbate supplied the complete flavin requirements of the chick from 2 to 5 weeks of age, determined by separate assay, on diets low in flavin (7).

A positive control group was included in each series of test groups from a single hatch of chicks. The positive control diet

<sup>3</sup> Obtainable from Vitab Products, Inc., of San Francisco, who kindly supplied it.

was Diet 80-G, 10 parts, plus rice bran filtrate<sup>3</sup> (4), 1 part. Rice bran filtrate was a crude aqueous extract of rice bran which had been shaken with fullers' earth, filtered, and concentrated to a thick syrup. 15 gm. of rice bran furnished 1 cc. of rice bran filtrate. The positive control diet supplied a generous excess of the filtrate factor.

During assay of purified concentrates of the filtrate factor, it was observed frequently that levels sufficient for the prevention of dermatitis during the 2 week assay period were not necessarily

TABLE I  
*Composition of Diets Corresponding to Fig. 1*

| Material   | Diet corresponding to Curve |                 |                 |                 |
|--|-----------------------------|-----------------|-----------------|-----------------|
|  | B                           | C               | D               | E               |
|  | <i>per cent</i>             | <i>per cent</i> | <i>per cent</i> | <i>per cent</i> |
| Heated yellow corn-meal.....   | 0                           | 0               | 55              | 53              |
| “ wheat middlings.....   | 25                          | 0               | 25              | 25              |
| “ commercial casein.....   | 6                           | 13              | 0               | 0               |
| Cow-peas.....  | 62                          | 0               | 0               | 0               |
| Rolled oats.....   | 0                           | 80              | 0               | 0               |
| Vacuum-dried beef round (dry weight).....                                  | 0                           | 0               | 13              | 0               |
| Vacuum-dried canned salmon (dry weight, entire contents of can dried)..... | 0                           | 0               | 0               | 15              |
| Minerals and vitamin supplements.  | Identical with Diet 80-G    |                 |                 |                 |

The positive control diet (Curve A) was described in the text. The basal diet (Curve F) was Diet 80-G. 13 parts of dried beef corresponded to 50 parts of the fresh material and 15 parts of dried salmon corresponded to 40 parts of the fresh material.

sufficient to produce the maximal growth response. Such sub-optimal levels did not completely prevent dermatitis if the feeding period was prolonged. Similar observations were made during the assay of feedingsuffs; *e.g.*, the chicks on Diet C (Fig. 1) were free from dermatitis at the end of the test, but had gained an average of only 54 gm. as compared with 80 gm. gained by the positive control group on Diet A.

Fig. 1 and Table I are illustrative of a typical assay. Table I shows how diets were adjusted to have a nutritive ratio similar to that of the basal diet.

The filtrate factor values of several feedingstuffs were given in a previous publication (1). The filtrate factor value is 100 divided by the per cent of the supplement which must be fed in the diet just to provide the level of filtrate factor for maximal growth on the heated diet under the conditions of the test. Hence a feedingstuff with a value of less than 1 cannot at any level supply the complete requirements of the chick for the vitamin. It is proposed to define a unit of the filtrate factor as follows: 1 unit of the filtrate factor is one-tenth of the amount which will just provide for maximal growth when fed daily to a chick 3 weeks old in conjunction with a heated diet under the conditions described above. It was found that chicks consumed about 10 gm. of diet daily at 3 weeks of age when receiving a heated diet which was adequately supplemented with a concentrated preparation of the filtrate factor. Hence the filtrate factor value of a preparation is the number of units present in 1 gm. of the preparation, assuming a daily food consumption of 10 gm. of test diet containing the preparation. It follows that the amount of the preparation which contains 1 unit of the factor is the reciprocal of the filtrate factor value of the preparation. From the growth data, therefore, it is possible to calculate (1) the filtrate factor value (the number of units of the vitamin in 1 gm. of the tested preparation) at one step. Table II expresses the results of some assays according to this notation. Supplements were fed at suboptimal levels and the filtrate factor values calculated from the growth data by the formula previously used (1).

Diet 80-G is probably not free from the filtrate factor. An allowance for this fact, and for any possible reserves stored in the bodies of the chicks, was made by subtracting the growth on the basal diet from the growth on the test diets and on the positive control diet when the filtrate factor value was calculated (1). This correction was more important when the supplement had only a small growth-promoting effect. If the filtrate factor value of a supplement was less than 0.2, growth promotion by the supplement was too small to bear a consistent relation to its filtrate factor content. Such supplements are listed in Table II as having a value of less than 0.2.

*Development of Gizzard Erosions in Chicks during Filtrate Factor Assay*—Gizzard erosions; previously described by McFarlane and

TABLE II

*Filtrate Factor Values of Some Feedingstuffs and Human Foods*

Some of the foods were dried before being fed, but all calculations are on the basis of undried weight.

| Material                                   | Amount fed<br>in ration | Filtrate<br>factor value | Mean filtrate<br>factor value |
|--|-------------------------|--------------------------|-------------------------------|
|  | <i>per cent</i>         | <i>units per gm.</i>     | <i>units per gm.</i>          |
| Rice bran extract, Type II (by volume)*    | 3                       | 20                       | 20                            |
| Bakers' Yeast 3 (unirradiated)†            | 3                       | 15                       |                               |
|  | 4                       | 21                       | 18                            |
| “ “ 3 (after irradiation)†                 | 3                       | 18                       |                               |
|  | 4                       | 19                       | 18                            |
| Peanut meal‡                               | 25                      | 3.2                      |                               |
|  | 25                      | 3.8                      | 3.5                           |
| Soy bean Meal 1                            | 20                      | 1.2                      |                               |
|  | 25                      | 1.6                      | 1.4                           |
| “ “ “ 2‡                                   | 20                      | 0.7                      |                               |
|  | 25                      | 0.6                      | 0.6                           |
| Cottonseed meal‡                           | 20                      | 1.3                      |                               |
|  | 25                      | 0.8                      | 1.0                           |
| Sesame meal‡                               | 20                      | 0.4                      |                               |
|  | 25                      | 0.4                      | 0.4                           |
| Linseed “ ‡                                | 10                      | <0.2                     | <0.2                          |
|  | 25                      |                          | Injurious                     |
| Coconut “ ‡                                | 15                      | <0.2                     |                               |
|  | 15                      | <0.2                     | <0.2                          |
| Babassu “ ‡                                | 15                      | <0.2                     | <0.2                          |
| Hexane-extracted wheat germ                | 30                      | 0.7                      | 0.7                           |
| Ground white milo                          | 58                      | 0.6                      |                               |
|  | 80                      | 0.7                      | 0.6                           |
| Rice Bran 2                                | 30                      | 1.9                      | 1.9                           |
| Alfalfa leaf meal                          | 10                      | 1.3                      | 1.3                           |
| Beef round, dried at 70° in vacuum oven    | 25                      | 0.8                      |                               |
|  | 50                      | 0.5                      | 0.7                           |
| Canned salmon, dried at 70° in vacuum oven | 40                      | 0.7                      |                               |
|  | 40                      | 0.5                      | 0.6                           |
| Onions, dried at 50°                       | 38                      | <0.2                     |                               |
|  | 58                      | <0.2                     | <0.2                          |

\* Obtainable from Vitab Products, Inc., of San Francisco, who kindly supplied it.

† Supplied by Standard Brands, Inc., of New York, by the kindness of Dr. C. A. Smith.

‡ Supplied by the Poultry Producers of Central California, through the courtesy of Dr. George Kernohan.



TABLE II—*Concluded*

| Material                        | Amount fed<br>in ration | Filtrate<br>factor value | Mean filtrate<br>factor value |
|---------------------------------|-------------------------|--------------------------|-------------------------------|
|                                 | <i>per cent</i>         | <i>units per gm.</i>     | <i>units per gm.</i>          |
| Carrots, dried at 50°           | 58                      | 0.2                      |                               |
|                                 | 116                     | <0.2                     | <0.2                          |
| Canned green peas, dried at 50° | 54                      | <0.2                     |                               |
|                                 | 54                      | <0.2                     | <0.2                          |
| Fresh " " " " 50°               | 110                     | 0.4                      | 0.4                           |
| Dried " " (split peas)          | 25                      | 1.4                      |                               |
|                                 | 40                      | 1.6                      | 1.5                           |
| Cow-peas                        | 62                      | 1.2                      |                               |
|                                 | 62                      | 1.4                      | 1.3                           |
| Navy beans, raw                 | 62                      | Injurious                |                               |
| " " autoclaved                  | 62                      | <0.2                     | <0.2                          |
| Rolled oats                     | 80                      | 0.8                      |                               |
|                                 | 50                      | 0.8                      | 0.8                           |
| Egg white, boiled 30 min.       | 12                      | <0.2                     |                               |
|                                 | 50                      | 0.2                      |                               |
|                                 | 80                      | <0.2                     | <0.2                          |
| " yolk, " 30 "                  | 14                      | 3.7                      |                               |
|                                 | 10                      | 4.2                      | 4.0                           |

coworkers (8) and by Almquist and Stokstad (9, 10), were usually noted in chicks on Diet 80-G, even when this was supplemented with high levels of the filtrate factor, as in the case of the positive control diet. Extensive gizzard erosions were found in chicks which had almost tripled in weight during the 2 week assay period. It appeared that gizzard erosions had no effect upon growth. This has already been noted by Almquist and Stokstad (9).

Irradiation of bakers' Yeast 3 was carried out by the Fleischmann Laboratories in their usual process for making vitamin D yeast. Apparently no destruction of the filtrate factor took place. The oil meals were ordinary commercial products used in poultry rations. The foods which were dried were incorporated with heated corn-meal before drying. Some of the foods had a high moisture content.

*Extraction of the Filtrate Factor*—Acidified hot or cold water readily and thoroughly extracted the filtrate factor from such feedingstuffs as wheat bran, rice bran, and alfalfa meal. The factor could not be detected in sardine meal, but it was present

in the stick water pressed from the sardines in the manufacture of sardine meal. Thus the filtrate factor is readily extracted by water. This property contrasts with the behavior of "vitamin B<sub>6</sub>" which was found by Birch and György (11) to be extracted with considerable difficulty from wheat germ and fish.

*Resistance of Filtrate Factor to Benzoylation*—A concentrate of the filtrate factor was prepared by removing inert matter from liver filtrate (4). The concentrate was treated twice with benzoyl chloride at 0° and pH about 8.6. The pH was then raised to about 9.4, and the solution was extracted with ether. A yellowish oil was removed. The solution was then acidified and shaken with ether to remove benzoic acid. The aqueous phase and the original concentrate were assayed with chicks. The results showed that no diminution in biological potency had occurred as a result of treatment with benzoyl chloride.

*Solubility of Filtrate Factor in Ethanol*—A concentrated solution of the filtrate factor, prepared by extraction of liver filtrate with isoamyl alcohol as previously described (3), was dried by repeatedly adding 95 per cent ethanol and evaporating under reduced pressure, followed by similar treatments with absolute ethanol. Finally a mass of crystals separated, which were removed by filtration and assayed with chicks. The supernatant liquid was evaporated to dryness, the distillate was collected, and the residue assayed. The distillate had a specific gravity corresponding to 99.5 per cent ethanol. The crystals were completely inactive, and the residue from the supernatant liquid was potent.

*Behavior towards Phosphotungstic Acid*—A concentrated solution of the filtrate factor, prepared from liver filtrate, was assayed and found to have a value of 75 units per gm. 9 cc. of sulfuric acid were added to 150 cc., followed by 50 gm. of phosphotungstic acid dissolved in a little hot water. The solution was allowed to stand at 0° overnight, and the precipitate was separated by filtration from the supernatant liquid. The precipitate was not washed. The two fractions were freed from phosphotungstic acid by treatment with barium hydroxide, and each was brought to 150 cc. The precipitable fraction was found to have a value of 5, and the non-precipitable fraction a value of 39 units per gm. Hence the treatment resulted in a loss of potency, but most of the vitamin was present in the filtrate from phosphotungstic acid.

## DISCUSSION

The demonstration of two new factors necessary for the rat, in the vitamin B complex, in addition to vitamin B and flavin (12), has made it difficult to interpret recent work on the vitamin B complex, particularly with regard to vitamin B<sub>6</sub> (11), which is probably a mixture of the two new factors. The nature of the deficiency produced in chicks by the heated diet, on the other

TABLE III

*Comparison of Filtrate Factor Content of Certain Foods with Pellagra-Preventive Values of Similar Foods As Determined by Goldberger and Coworkers*

The weights are on an undried basis unless otherwise stated.

| Food                               | Amount containing 1 unit of filtrate factor (= 1 + filtrate factor value) | Human pellagra-preventive value | Daily amount fed in human pellagra test | Bibliographic reference to human pellagra or black tongue test |
|------------------------------------|---|---------------------------------|---|--|
|                                    | gm.   |                                 | gm.                                     |  |
| Dried egg yolk.....                | 0.13  | Fair (black tongue)             |   | 13   |
| Peanut meal.....                   | 0.3   | Good                            | 200                                     | 14   |
| Dried green peas (split peas)..... | 0.6   | Fair                            | 360                                     | 15   |
| Cow-peas.....                      | 0.8   | "                               | 178                                     | 16   |
| Rolled oats.....                   | 1.2   | None (black tongue)             |   | 17   |
| Kale.....                          | 1.2   | Good                            | 534                                     | 18   |
| Wheat germ, fat-extracted...       | 1.4   | "                               | 150                                     | 16   |
| Fresh beef round...                | 1.5   | "                               | 200                                     | 19, 20   |
| Whole corn-meal.....               | 1.7   | None                            | 270                                     | 18   |
| Canned Alaska salmon.....          | 1.7   | Good                            | 168                                     | 21   |
| Carrots.....                       | >5.0  | Slight                          | 450                                     | 22   |
| Mature onions.....                 | >5.0  | None                            | 525                                     | 23   |
| Canned green peas.....             | >5.0  | Good                            | 450                                     | 24   |

hand, appears to be uncomplicated, and all observations have indicated that a single factor, the filtrate factor, is the sole deficiency.

A number of the materials included in Table II have been examined for their human pellagra-preventive value by Goldberger and his coworkers. Table III compares the filtrate factor content of some of these foods with the pellagra-preventive values of simi-

lar foods as determined by the researches of Goldberger and his collaborators.

Koehn and Elvehjem (25) have cured black tongue in dogs with a concentrate of the filtrate factor (vitamin B<sub>2</sub> of Elvehjem and Koehn (2)) freed from flavin by treatment with fullers' earth. From this, these workers deduced that their vitamin B<sub>2</sub> (filtrate factor) was the anti-black tongue factor, and therefore probably the P-P factor. Fouts and coworkers (26) have reported the successful treatment of human pellagrins with a concentrate of the filtrate factor (12) free from lactoflavin and the rat antidermatitis factor. It may be seen, however, from Table III that striking differences exist between the P-P values of foods and their filtrate factor values. The cereal grains, including corn, all supply appreciable amounts of the filtrate factor. It is of interest to note that corn is about as good a source of the filtrate factor as wheat, oats, or barley. Cow-peas, which furnish only small amounts of the P-P factor, were superior as a source of the filtrate factor to several foods which have been shown by Goldberger and his associates to be active in the prevention or cure of human pellagra, such as beef, wheat germ, canned salmon, and kale. The low filtrate factor potency of beef is especially worthy of note in view of its well established P-P value.

#### SUMMARY

1. Details are described of a revised method for the assay of the filtrate factor (a water-soluble vitamin belonging to the vitamin B complex and preventing a dietary dermatitis in chicks), and a unit is proposed consisting of one-tenth of the optimal daily requirement of the chick under the conditions described.

2. Gizzard erosions in chicks were not prevented by the filtrate factor. Growth did not appear to be affected by gizzard erosions.

3. The distribution of the factor in certain feedingstuffs and human foods is reported.

4. The filtrate factor was readily removed from certain feedingstuffs by extraction with water.

5. The factor in aqueous solution was not destroyed by treatment with benzoyl chloride. The factor was soluble in 99.5 per cent ethanol. It was present in the filtrate from treatment with phosphotungstic acid, although some loss occurred.

6. It is pointed out that marked differences exist between the distribution of the filtrate factor as determined in this investigation and the distribution of the human pellagra-preventive factor as noted by Goldberger and coworkers.

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# THE LIPIDS OF CONNECTICUT SHADE-GROWN TOBACCO SEED

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As pointed out by Roberts and Schuette (1) there appears to be not only an inadequate amount of data on the composition of tobacco seed oils, but there are certain contradictions in some of the data available. Papers appearing since that of the authors referred to above are principally concerned with determination of the usual fat constants, and give little information as to the exact constituents of tobacco seed oil (2).

The purposes of the present investigation have been to determine the constituents of oils from seeds of Connecticut shade-grown tobacco for comparison with oils from other sections of this country and elsewhere, and to determine the amount of phospholipids present. It was found that the oils were an ordinary mixture of triglycerides which yielded palmitic, stearic, oleic, and linoleic acids and glycerol upon saponification; that some solid fatty acid or acids higher than  $C_{18}$  could be isolated in small amount from the saponification mixture, although similar acids were not found in the oils of Wisconsin-grown seeds (1); that the unsaponifiable matter was sitosterol; and that the phospholipids appeared to be an impure mixture of lecithin and cephalin, and represented only about 0.1 per cent of the total fats, which were not examined because of the small amount available.

## EXPERIMENTAL

The seed used for this investigation was provided from the 1931 crop through the courtesy of H. B. Vickery, T. R. Swanback, and the facilities of the Tobacco Substation of the Connecticut Agricultural Experiment Station, at Windsor.

The seed was crushed with mortar and pestle and extracted at room temperature under carbon dioxide for 1 week periods with freshly distilled solvents saturated with carbon dioxide. Three such extractions with acetone gave 1446 gm. of ether-soluble material from 4.5 kilos of seed. The acetone extractions were followed by one 50 per cent aqueous alcohol extraction (to facilitate the liberation of phospholipids from the cells) and two alcohol-ether extractions. The last three extractions yielded 167 gm. of ether-soluble material. The total fats weighed 1613 gm. and constituted 35.8 per cent of the original weight of the seed. The acetone-extracted fats were a clear, light yellow fluid, whereas the combined alcohol-water- and alcohol-ether-extracted fats were dark brown but quite fluid. The two oils were examined separately.

*Acetone-Extracted Lipids*—483.7 gm. of the acetone-extracted lipids, having an iodine number (Hanus) of 133.7, were saponified with alcoholic KOH. No unsaponifiable matter was found, although the aqueous soap solution was repeatedly extracted with ether. 453.7 gm. of fatty acids were obtained, and from the aqueous residue, after removal of potassium chloride, 40 gm. of glycerol were separated, distilling at 145–155° at 2 mm., and forming a tribenzoate (3) melting at 76–76.5°.

448.4 gm. of the mixed fatty acids were separated by the lead soap-ether method (4) into 63.3 gm. of solid acids (14.5 per cent) and 373.9 gm. of liquid acids (85.5 per cent) having an iodine number (Hanus) of 149.4.

*Procedure for Examination of Fatty Acids*—The fatty acids were converted into their methyl esters and the esters were fractionated by distillation at 1 to 1.5 mm. The melting point and index of refraction at 55° were taken as criteria of purity for the esters. The purified esters were saponified and the acids were isolated. After crystallization the acids were identified by melting point, mixed melting point, and molecular weight by titration. From the indices of refraction and the amounts of the ester fractions, the per cent composition of the esters was estimated. For brevity, many of these details are omitted.

*Solid Fatty Acids of Acetone Extract*—After purification by Twitchell's method (5) the solid acids were methylated and the esters fractionated, yielding methyl palmitate, methyl stearate,

and a small amount of the methyl ester of some acid of higher molecular weight.

The methyl palmitate melted at  $28^{\circ}$ ,  $n_D^{25} = 1.4262$ . The free acid melted at  $62^{\circ}$ , showed no depression in melting point when mixed with an authentic sample of palmitic acid, and had a molecular weight by titration with alcoholic KOH of 256.6.

The methyl stearate melted at  $37.5^{\circ}$ ,  $n_D^{25} = 1.4298$ . The free acid melted at  $69^{\circ}$ , showed no depression in melting point when mixed with an authentic sample of stearic acid, and had a molecular weight by titration of 285.1.

The residue from the distillation of 51.5 gm. of the above esters, weighing 2.60 gm., was saponified and the recovered acid twice crystallized from acetone. The acid then melted at  $62-63^{\circ}$ , solidified at  $61.5^{\circ}$ , and remelted at  $63^{\circ}$ . The molecular weight by titration was 313.8. Five recrystallizations from ethyl acetate and four more from ethanol produced no change in the melting point. The acid was combined with that from the recrystallization mother liquors and reconverted to the methyl ester. After distillation, the esters were again distilled and the acids from the three final fractions had the following properties.

| Fraction No. | Weight | M p. of acid        | Mol. wt |
|--------------|--------|---------------------|---------|
|              | gm.    | $^{\circ}\text{C.}$ |         |
| 5            | 0.25   | 61.5-62             | 297.1   |
| 6            | 0.15   | 66.5-67.5           | 332.8   |
| 7            | 0.09   | 75 -76              | 398     |

The amounts available did not permit further identification, but the melting point and molecular weight of Fraction 7 clearly indicate the presence of a small amount of some acid or acids higher than  $\text{C}_{18}$ , probably not more than 1 per cent of the total solid acids.

From the indices of refraction of the esters the solid acids were estimated to be 66.3 per cent palmitic acid and 33.7 per cent stearic acid.

*Liquid Fatty Acids, Acetone Extract*—142 gm. of the liquid acids from the acetone extract were hydrogenated in the presence of platinum oxide (6). The reduced acids were found to contain no liquid saturated acids, and consisted of pure stearic acid. The



acids were, however, converted into their methyl esters and fractionally distilled to give forty-one fractions of approximately equal amounts with melting points very close to one another (36.5–39°) and refractive indices in agreement with that for methyl stearate. The residue from this fractionation weighed 4.14 gm. and was found to be practically pure methyl stearate. The once crystallized acid prepared from the residual esters melted at 69° and had a molecular weight of 285.4. Evidently there were no acids higher than  $C_{18}$  in the original liquid acids.

*Bromination of Liquid Acids, Acetone Extract*—16.79 gm. of the liquid acids in petroleum ether (30–60°) were treated at –15° in the dark with a small excess of a petroleum ether solution of bromine. After standing several hours at this temperature, the reaction mixture was filtered and washed with cold petroleum ether. 17.6 gm. of insoluble bromides and 15.17 gm. of soluble bromides were obtained. The insoluble bromides were crystallized from ethanol and found to melt at 114° (linoleic tetrabromide; melting point, 114°) and to have a bromine content (Carius) of 53.16 per cent. Theory for  $C_{18}H_{32}Br_4O_2$ , 53.28.

Since only tetrabromides and dibromides were found, the composition of the original liquid acids may be calculated from the iodine number to be 34.8 per cent oleic acid and 65.2 per cent linoleic acid.

*Alcohol-Ether-Extracted Fat*—By the same methods that were used for examination of the acetone-extracted fats, the alcohol-ether extract was found to contain 1.11 gm. of acetone-insoluble material (phospholipids), 2.4 gm. of unsaponifiable matter, and glycerides quite similar to those found in the acetone extract.

*Phospholipids*—Although too small in amount for any extended examination, the acetone-insoluble material was precipitated three times from ether solution by the addition of acetone, washed with acetone, and dried. In the crude state in which it was thus obtained the phospholipid fraction was found to contain 3.01 per cent N and 1.60 per cent P, and was obviously quite impure.

*Unsaponifiable Matter*—After four recrystallizations from ethanol and two from ethyl acetate, the unsaponifiable matter was obtained in flat white crystals melting at 138°,  $[\alpha]_D^{27} = -38.6^\circ$  in chloroform. The 3,5-dinitrobenzoate was prepared from the corresponding acid chloride and the sterol in pyridine. After one

crystallization from acetone the derivative melted sharply at 203°. All of the properties mentioned are in agreement with the corresponding properties of sitosterol.

*Fatty Acids*—After saponification and examination in the usual way the fatty acids were found to be 25.8 per cent solid acids (lead soap-ether method) and 74.2 per cent liquid acids having an iodine number (Hanus) of 165.0.

The solid acids were 45 per cent palmitic acid and 55 per cent stearic acid, exclusive of a trace of some acid or acids higher in molecular weight than stearic acid.

The liquid acids were hydrogenated and the reduced acids were found to be pure stearic acid. Bromination of the liquid acids produced only dibromides and tetrabromides. Hence, the composition of the liquid acids as calculated from the iodine number was 17.7 per cent oleic acid and 82.3 per cent linoleic acid.

#### SUMMARY

Connecticut shade-grown tobacco seed was found to contain 35.8 per cent of lipids extractable with acetone and alcohol-ether. The lipids were found to consist principally of triglycerides, but also contained 0.15 per cent of sitosterol and 0.07 per cent of phospholipids.

The fatty acids obtained upon saponification of the glycerides were 9.8 per cent palmitic acid, 5.9 per cent stearic acid, 28.0 per cent oleic acid, and 56.3 per cent linoleic acid. Also found were small amounts of some solid acid or acids of molecular weight greater than stearic acid.

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# **A METHOD FOR PROTECTING THE IMIDAZOLE RING OF HISTIDINE DURING CERTAIN REACTIONS AND ITS APPLICATION TO THE PREPARATION OF *l*-AMINO-N-METHYLHISTIDINE**

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The particular difficulty in applying to histidine the ordinary method of preparing methyl-N-amino acids by methylation of the toluene-sulfonyl derivative is the sensitivity of the imino group of the imidazole ring to methylation. If this grouping could be covered during methylation by some protective grouping which could later be readily removed, the conventional methylation procedure might be permitted. This would enable one to obtain the active methylamino derivative directly from *l*-histidine and, since no opportunity for Walden inversion would be afforded, one would be assured of having the compound with the spatial configuration desired.

The only method available at the present time for the synthesis of amino-N-methylhistidine depends on the reaction of methylamine with  $\alpha$ -chloro- $\beta$ -imidazole-4-propionic acid (1), the latter being prepared by treatment of histidine with fuming HCl and NaNO<sub>2</sub> according to the directions of Windaus and Vogt (2) or by direct synthesis (3). In both the replacement of the amino group of histidine by chlorine and the replacement of the latter with methylamine, opportunity for a Walden inversion with the possibility of accompanying racemization exists. In fact Fargher and Pyman (1) isolated *dl*-methylhistidine even though they started with *l*-histidine. To obtain the active methylhistidine, corresponding in stereostructure to the naturally occurring series of amino acids, it would be necessary to resolve the inactive methylhistidine. When the yield which was to be expected in

the resolution was taken into consideration with the poor yields obtainable in the preparation of the *dl*-methylhistidine, it was realized that some other method of approach was needed.

It occurred to us that the imino hydrogen of the imidazole ring might react in liquid ammonia with metallic sodium and that the resulting compound might then react with benzyl chloride to yield a benzyl derivative. Furthermore, it seemed possible that this benzyl grouping might be subject to cleavage by reduction in liquid ammonia with metallic sodium, similarly to the cleavage of the benzyl grouping from benzylthiol ethers such as benzylcysteine (4), and benzylhomocysteine (5), and from benzyl esters such as carbobenzoxy-cysteine and carbobenzoxy-carnosine (4).

In exploring the possibilities of this reaction, histidine monohydrochloride was dissolved in liquid ammonia and 4 moles of metallic sodium were added, 1 mole for the carboxyl group, 1 for the molecule of water of crystallization in the histidine monohydrochloride, 1 for the HCl, and 1 for the imino hydrogen. A slight excess of benzyl chloride was then added. After the evaporation of the ammonia solution, the residue was dissolved in water and extracted with ether to remove any benzyl chloride. Upon bringing the solution to a pH of 8.0 a crystalline product (I) was obtained. The product after recrystallization from 70 per cent alcohol melted at 248–249° (corrected) and crystallized in plates. The material exhibited a specific rotation of  $[\alpha]_D^{24} = +20.5^\circ$  for a 2 per cent aqueous solution containing 1 equivalent of HCl. When the mother liquor from (I) was brought to pH 7.0 to 7.5, a second crystalline compound (II) was obtained, which, after recrystallization from water, melted at 193–195° (corrected). It crystallized in long needle-like crystals and had a specific rotation of  $[\alpha]_D^{24} = +34.5^\circ$  under the conditions used for (I). Both compounds failed to respond to the Pauly diazo reaction (6) as would be expected of compounds substituted on the imino N of the imidazole ring. On the other hand, (I) gave a positive ninhydrin reaction, whereas (II) gave a negative test, giving rise to the suspicion that perhaps the latter was substituted not only on the imidazole ring but also on the amino N as well. This was further supported by the fact that (II) did not give a Knoop bromine test (7). It has been reported that when

1 atom of the hydrogen of the amino group is substituted by a methyl group or as in carnosine by the  $\beta$ -alanyl radical, the bromine color test is negative (8). The other compound, which we suspected on the basis of the Pauly and ninhydrin reactions of being substituted only in the imidazole ring, gave a positive bromine test. The positive reaction was in keeping with this idea of its structure, since it had been found that the bromine test is positive with 1-methylhistidine obtainable from anserine (9). The analytical data were also in complete agreement with the above interpretations. The values agreed with the empirical formula of  $C_{13}H_{15}O_2N_3$  for (I) and  $C_{20}H_{21}O_2N_3$  for (II). We therefore concluded that the compounds are 1(or 3)-benzyl-L-histidine and amino-N-benzyl-1(or 3)-benzyl-L-histidine.

A yield of 48 per cent of the monobenzylhistidine and 22 per cent of the dibenzyl compound were obtained based on the histidine started with when 1.5 moles of benzyl chloride were used. When the benzyl chloride was decreased to 1.1 moles, the yield of the monobenzyl compound was increased to 57 per cent and the yield of the dibenzylhistidine was decreased to 8 per cent.

The obtaining of a dibenzyl derivative by this reaction was particularly interesting to us and somewhat surprising in that we had never had any intimation of benzylation or methylation of the  $\alpha$ -amino group in the preparation of benzylthiol and methylthiol derivatives of amino acids by the addition of benzyl chloride or methyl iodide to the sodium salts of the sulfhydryl amino acids in liquid ammonia. The difference may depend on the relative activities of the imino and the amino hydrogens in histidine, on the one hand, and of the sulfhydryl and the amino hydrogens in the sulfhydryl amino acid on the other.

To test the second part of our postulated approach, that is, the cleavage of the benzyl group from the imidazole ring, reduction of the monobenzyl derivative by metallic sodium in liquid ammonia was attempted. Histidine was obtained in excellent yield from the reaction mixture, demonstrating that the benzyl group-ing can be successfully removed in this manner. The toluenesulfonyl derivative of the 1(or 3)-benzyl-L-histidine was, therefore, prepared and methylated with methyl iodide.

In view of the fact that the toluenesulfonyl group can be removed by reduction with HI and phosphonium iodide, it was

deemed worth while to see if the grouping might be removed by the sodium-liquid ammonia reduction. If that were the case, the benzyl radical and the *p*-toluenesulfonyl group could be removed in one step. The reduction of *p*-toluenesulfonyl-1(or 3)-benzyl-*l*-histidine was first tried and histidine was isolated from the reduction mixture in excellent yield. In order to ascertain whether any racemization had taken place the histidine monohydrochloride was converted to the methyl ester dihydrochloride and the specific rotation was determined. This was done because histidine hydrochloride possesses such a small rotation. The rotation of the ester dihydrochloride agreed in value with the histidine started with and with the values recorded in the literature.

After the cleavage of the *p*-toluenesulfonyl group by reduction with metallic sodium had been established, the reduction of the *p*-toluenesulfonyl-N-methyl-1(or 3)-benzyl-*l*-histidine was attempted, and found to proceed as expected. After evaporation of the ammonia, the *l*-amino-N-methylhistidine was isolated from the residue as the dipicrate and the latter was converted to the monohydrochloride. The compound crystallized in plates and melted at 268° (corrected). An 88 per cent yield of the monohydrochloride was obtained from the reduction of the *p*-toluenesulfonyl-N-methyl-1(or 3)-benzyl-*l*-histidine. The methylhistidine gave a positive Pauly diazo reaction and a negative ninhydrin reaction. The free base was isolated as clusters of needles from the monohydrochloride through the use of  $\text{Ag}_2\text{SO}_4$  and  $\text{Ba}(\text{OH})_2$ . The free methylhistidine possessed a specific rotation of  $-13.5^\circ$  for a 1 per cent solution in water and melted at 266° (corrected). The dihydrochloride was also prepared.

This series of reactions makes readily available not only the active amino-N-methylhistidine corresponding in stereostructure to the naturally occurring series of amino acids, but introduces as well three new reactions which should find wide application. The benzylation reaction can without much doubt be extended to the introduction of alkyl radicals into imidazoles in general and perhaps to certain other similar ring nitrogen compounds. It may not be out of place even to suggest the extension of the reaction to arginine and other guanidine compounds. The combination of benzylation of the imidazole ring and subsequent

removal by reduction introduces a new method of protecting the imidazole ring in various reactions, the many possible applications of which should be apparent. Finally the splitting of the toluenesulfonyl group may find considerable application in peptide synthesis, and offers no doubt, at least in certain instances, an alternative to the carbobenzoxy method of peptide synthesis. Furthermore where particular solubilities or crystalline properties are desired, a choice of sulfonyl derivatives will be afforded, since the naphthalenesulfonyl, benzenesulfonyl, and possibly certain other sulfonyl derivatives would be expected to react in similar manner.

#### EXPERIMENTAL

*Preparation of 1(or 3)-Benzyl-L-Histidine*—20 gm. of histidine monohydrochloride were placed in 200 cc. of dry liquid ammonia in a 500 cc. 3-neck round bottom flask fitted with a mercury seal mechanical stirrer. Small pieces of sodium were added to the mixture, which was kept in a trichloroethylene-solid  $\text{CO}_2$  bath, until a permanent blue color remained, indicating the presence of excess sodium. 9.0 gm. of sodium were required. The blue color was discharged by the addition of a small amount of histidine, leaving a light brown solution containing a white precipitate. 12 cc. of benzyl chloride (1.1 moles) were added dropwise from a small separatory funnel while the mixture was vigorously stirred. The stirring was continued for 30 minutes, after which the ammonia was allowed to evaporate spontaneously. The flask was finally evacuated to aid in the removal of the excess ammonia. The residue was dissolved in 100 cc. of ice water and the solution immediately extracted with ether to remove the excess benzyl chloride. Ether and any excess of ammonia which remained were removed by aeration. The solution was filtered and dilute  $\text{H}_2\text{SO}_4$  was added slowly with stirring until precipitation began (pH 8.0 to 8.5). The solution was allowed to stand in the ice box for 3 hours and was then filtered. The precipitate was recrystallized from 70 per cent ethyl alcohol. The compound crystallized in plates and melted at  $248\text{--}249^\circ$  (corrected) with softening at  $243^\circ$ . A 2 per cent solution containing 1 equivalent of  $\text{HCl}$  gave a rotation of  $[\alpha]_D^{24} = +20.5^\circ$ . 13.4 gm. of the recrystallized product were obtained, representing



about 57 per cent of the theoretical yield. The compound gave a positive ninhydrin reaction and a positive Knoop bromine reaction, but did not respond to the Pauly diazo test. It was soluble in acid, alkali, and in hot water, but was insoluble in acetone, ether, or absolute alcohol. For analysis the compound was recrystallized once more and dried *in vacuo* at  $110^{\circ}$  over  $P_2O_5$ . The product had the following composition.

|                        |             |                          |
|------------------------|-------------|--------------------------|
| $C_{13}H_{15}O_2N_3$ . | Calculated. | C 63.64, H 6.17, N 17.14 |
|                        | Found.      | " 64.09, " 6.12, " 16.94 |

*Isolation of Amino-N-Benzyl-1(or 3)-Benzyl-l-Histidine*—The mother liquor from the 1(or 3)-benzyl-l-histidine precipitation was brought to pH 7.0 to 7.5 with dilute  $H_2SO_4$  and allowed to stand in the ice box overnight. 2.9 gm. of material were obtained. This product was recrystallized from hot water and 2.6 gm. of needles with a melting point of  $193-195^{\circ}$  (corrected) were obtained, representing 7 per cent of the histidine. A 2 per cent solution containing 1 equivalent of HCl gave a rotation of  $[\alpha]_D^{24} = +34.5^{\circ}$ . These needles gave neither the Pauly diazo test, the Knoop bromine reaction, nor the ninhydrin reaction. The compound was soluble in hot water, acid, alkali, and hot absolute alcohol, but insoluble in ether and acetone. For analysis the compound was recrystallized once more and was dried *in vacuo* at  $110^{\circ}$  over  $P_2O_5$ . It had the following composition.

|                        |             |                          |
|------------------------|-------------|--------------------------|
| $C_{20}H_{21}O_2N_3$ . | Calculated. | C 71.60, H 6.31, N 12.54 |
|                        | Found.      | " 71.27, " 6.11, " 12.20 |

*Preparation of N-p-Toluenesulfonyl-1(or 3)-Benzyl-l-Histidine*—10 gm. of 1(or 3)-benzyl-l-histidine were dissolved in 91 cc. of 2 N NaOH. 18.6 gm. of finely pulverized *p*-toluenesulfonyl chloride were added and the mixture was shaken vigorously for 45 minutes. The small residue of unchanged *p*-toluenesulfonyl chloride was filtered and the solution slowly acidified to litmus with dilute  $H_2SO_4$ . Vigorous scratching brought the precipitation of 12.2 gm. of rod-like crystals. Further acidification of the mother liquor caused the formation of an oil. The latter was crystallized from butyl alcohol. A total of 13.4 gm. of product representing about 82 per cent of the theoretical yield were obtained. This material was sufficiently pure to use in the next

step. For analysis the material was recrystallized from butyl alcohol. The recrystallized material had a melting point of  $198^{\circ}$  (corrected), was soluble in ethyl alcohol, butyl alcohol, acid, and alkali, and was insoluble in water, ether, and petroleum ether. The product, dried *in vacuo* over  $P_2O_5$  at  $110^{\circ}$ , had the following composition.

$C_{18}H_{21}O_4N_3S$ . Calculated, N 10.53; found, N 10.56

*Preparation of p-Toluenesulfonyl-N-Methyl-1(or 3)-Benzyl-L-Histidine*—20 gm. of N-*p*-toluenesulfonyl-1(or 3)-benzyl-L-histidine were dissolved in 210 cc. of 0.5 N NaOH, 3.43 cc. of methyl iodide were added, and the mixture was shaken in a pressure bottle at  $68-70^{\circ}$  for 40 minutes. The reaction mixture was cooled and slowly acidified to pH 4. 8.7 gm. of hexagonal plates separated. Further acidification caused the formation of an oil, which, after being taken up in alkali and reacidified, gave 3.5 gm. of starting material. The yield of the methylated compound, based on the starting material actually used, was 51 per cent. The compound had a melting point of  $118-122^{\circ}$  (corrected). It was soluble in acid, alkali, ethyl alcohol, and butyl alcohol, and was insoluble in ether. For analysis the material was dissolved in alkali and reprecipitated by acidification and the precipitate was thoroughly washed with water. The compound dried over  $P_2O_5$  at  $64^{\circ}$  *in vacuo* had the following composition.

$C_{21}H_{25}O_4N_3S$ . Calculated, N 10.17; found, N 9.93

*Reduction of N-p-Toluenesulfonyl-1(or 3)-Benzyl-L-Histidine*—4 gm. of N-*p*-toluenesulfonyl-1(or 3)-benzyl-L-histidine were placed in 25 cc. of dry liquid ammonia and reduced in the usual manner. 1.55 gm. of sodium were sufficient to produce a permanent blue color. The excess sodium was discharged with  $NH_4Cl$  and the ammonia removed as described above. The white residue which remained was dissolved in 20 cc. of water and the resulting solution was thoroughly aerated to remove the ammonia. The solution was then acidified to Congo red with dilute HCl, and the solution was extracted with ether. Water was added to bring the volume to 200 cc. and a solution of 16 gm. of  $HgCl_2$  in 80 cc. of hot 95 per cent ethyl alcohol was added with stirring. Saturated  $Na_2CO_3$  solution was then added to bring the pH of the

reaction mixture to 7.0 to 7.5. The precipitate was removed by filtration and was washed twice by suspension in water. The mercury was then removed as HgS. The aqueous solution was evaporated to dryness, and traces of water were removed by repeated distillation with absolute methyl alcohol. The solid which remained was dissolved in 30 cc. of hot absolute methyl alcohol and the solution was saturated with dry HCl gas. When the solution was cooled the histidine methyl ester dihydrochloride precipitated. It was purified by dissolving it in 20 cc. of absolute methyl alcohol and again saturating the solution with HCl. 0.65 gm. of histidine methyl ester dihydrochloride (27 per cent) was obtained. This showed a rotation of  $[\alpha]_D^{25} = +10.5^\circ$  and a melting point of  $197^\circ$  (corrected).

*Reduction of p-Toluenesulfonyl-N-Methyl-1(or 3)-Benzyl-L-Histidine and Isolation of l-Amino-N-Methylhistidine Monohydrochloride*—10 gm. of toluenesulfonyl-N-methyl-1(or 3)-benzyl-l-histidine were dissolved in 50 cc. of dry liquid ammonia. Small pieces of sodium were added with shaking until a permanent blue color remained. 3.75 gm. of sodium (6 equivalents) were used.  $\text{NH}_4\text{Cl}$  was added to the reaction mixture to discharge the excess sodium, leaving a clear solution with a white precipitate. The ammonia was allowed to evaporate and the container finally evacuated. The precipitate was dissolved in 100 cc. of water and the aqueous solution thoroughly aerated. The solution was neutralized to litmus and extracted with ether. The solution was then evaporated to dryness *in vacuo* and the dry material dissolved in 390 cc. of hot water containing 19.0 gm. of picric acid. On standing in the ice box overnight 16.5 gm. of the dipicrate precipitated in prismatic needles with a melting point of  $61^\circ$  (corrected).

The picrate was decomposed by boiling with 210 cc. of 3 per cent hydrochloric acid and the picric acid was removed by extracting the mixture three times with 50 cc. portions of benzene and four times with 50 cc. portions of ether. After the aqueous solution was treated with norit, it was evaporated to about 10 cc. and taken up in 50 cc. of absolute alcohol. 6.0 cc. of aniline were added with stirring, causing the precipitation of 4.4 gm. (88 per cent) of l-amino-N-methylhistidine monohydrochloride. For analysis the monohydrochloride was recrystallized by dis-

solving in the minimum amount of hot water and adding 4 volumes of absolute alcohol. The plates melted with decomposition at  $268^{\circ}$  (corrected) and gave the following analysis.

$C_7H_{11}O_2N_2 \cdot HCl$ . Calculated, N 20.44; found, N 20.12

*Preparation of l-Amino-N-Methylhistidine Dihydrochloride*—A small amount of the monohydrochloride was dissolved in dilute HCl and concentrated to small volume. On standing in the ice box overnight the dihydrochloride crystallized in large masses of diamond-shaped crystals. The melting point was found to be  $124$ – $127^{\circ}$  (corrected). The compound, containing 1 molecule of water of crystallization, had the following composition.

$C_7H_{11}O_2N_2 \cdot 2HCl \cdot H_2O$ . Calculated, N 16.16; found, N 15.82

*Preparation of l-Amino-N-Methylhistidine*—0.7 gm. of *l*-amino-N-methylhistidine monohydrochloride was dissolved in 30 cc. of water containing 0.55 gm. of silver sulfate. The precipitated silver chloride was filtered and the excess silver removed as silver sulfide. The sulfate was quantitatively removed with barium hydroxide and the filtered solution concentrated to 2 cc. 15 cc. of absolute alcohol were added and the solution allowed to stand in the ice box for several hours. 0.45 gm. of clusters of rod-like plates were obtained with a melting point of  $247^{\circ}$  (corrected). After the material was recrystallized three times from hot water with the addition of several volumes of ethyl alcohol, it exhibited a melting point of  $266^{\circ}$  (corrected) and a rotation of  $[\alpha]_D^{20} = -13.5^{\circ}$  for a 1 per cent aqueous solution. The air-dried free base contained 1 molecule of water of crystallization and had the following composition.

$C_7H_{11}O_2N_2 \cdot H_2O$ . Calculated. C 44.89, H 7.00, N 22.46  
Found. " 44.97, " 6.82, " 22.40

The authors wish to thank Mr. C. Rodden, microanalyst of this laboratory, for carrying out the microanalyses.

#### SUMMARY

*l*-Histidine, when treated in liquid ammonia with metallic sodium and benzyl chloride, has been shown to yield mainly a

monobenzyl derivative in which the benzyl group is attached to the imidazole nitrogen. Small amounts of a dibenzyl compound, amino-N-benzyl-1(or 3)-benzyl-*l*-histidine, were also isolated from the reaction mixture.

Debenzylation of the monobenzyl derivative, 1(or 3)-benzyl-*l*-histidine, has been accomplished by reduction in liquid ammonia by metallic sodium. The combination of benzylation and debenzylation affords a method of protecting the imidazole ring during certain reactions.

It has been shown that the *p*-toluenesulfonyl group can also be removed by reduction in liquid ammonia with metallic sodium. The extension of this reaction to other possible uses has been pointed out.

Advantage was taken of the above reactions in devising a method for the preparation of *l*-amino-N-methylhistidine. The *p*-toluenesulfonyl derivative of 1(or 3)-benzyl-*l*-histidine was methylated with methyl iodide in the usual manner, and the resulting *p*-toluenesulfonyl-N-methyl-1(or 3)-benzyl-*l*-histidine was converted to *l*-amino-N-methylhistidine by removing the benzyl and the toluenesulfonyl groups in one step by the sodium-liquid ammonia reduction. An 88 per cent yield was obtained in the reduction. An over-all yield of 21 per cent of the theoretical amount of *l*-methylhistidine on the basis of the *l*-histidine started with was obtained.

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## SYNTHETIC NUCLEOSIDES

### V. THEOPHYLLINE-*d*-ALLOMETHYLOSIDE

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Interest in the synthesis of nucleosides containing the hexomethyloses as the sugar component has been principally due to the similarity of these sugars with the pentoses. Thus, Fischer and von Fodor<sup>1</sup> successfully synthesized theophylline-*l*-rhamnoside and theobromine-*l*-rhamnoside. More recently  $\beta$ -theophylline-*d*-isorhamnoside(*d*-glucomethylose) has been synthesized<sup>2</sup> by the reduction of theophylline-tetraacetyl- $\beta$ -*d*-glucoside-6-bromohydrin followed by deacetylation.

The recent synthesis of *d*-allomethylose<sup>3</sup> by an inversion of *l*-rhamnose places this heretofore rare sugar among those easily accessible. Also because of the similarity of this sugar to *d*-ribose, the nucleosides which may be derived from it are of special importance. Accordingly, a report of the work leading to the synthesis of theophylline-*d*-allomethyloside is made at this time.

Acetylation of *d*-allomethylose with acetic anhydride yields tetraacetyl *d*-allomethylose. The preparation of the acetobromo sugar from this substance may be readily accomplished with acetic acid-hydrogen bromide in the conventional manner but owing to the instability of this derivative it could not be obtained in crystalline form. However, the bromo sugar may be easily condensed with silver theophylline in anhydrous toluene to yield theophylline-triacetyl-*d*-allomethyloside combined with 1 molecule of toluene of crystallization (m.p. 140°). This addition

<sup>1</sup> Fischer, E., and von Fodor, K., *Ber. chem. Ges.*, **47**, 1058 (1914).

<sup>2</sup> Fischer, E., Helferich, B., and Ostmann, P., *Ber. chem. Ges.*, **53**, 873 (1920).

<sup>3</sup> Levene, P. A., and Compton, J., *J. Biol. Chem.*, **116**, 169 (1936).

compound is rather unusual for this class of substances and apparently is very stable at room temperature. Upon heating above 100° under a high vacuum for a short time, however, the toluene is completely removed without apparent change in crystal structure. The product obtained in this manner, theophylline-triacetyl-*d*-allomethyloside (m.p. 217–218°), may be easily recrystallized from anhydrous methyl alcohol without change in melting point but upon recrystallization from toluene the addition compound is again formed.

The deacetylation of theophylline-triacetyl-*d*-allomethyloside yields crystalline theophylline-*d*-allomethyloside with a melting point of 167–168°. In general the acetyl derivatives of the nucleosides have a lower melting point than the nucleoside itself. In the present case there is thus an exception to this rule in that the nucleoside has a lower melting point than its triacetyl derivative. Because of this unusual behavior, the possibility of some structural change accompanying deacetylation was considered. That such had not occurred, however, was shown by the fact that the nucleoside upon acetylation with acetic anhydride yielded the original theophylline-triacetyl-*d*-allomethyloside (m.p. 217–218°).

From a study of the acid hydrolysis of theophylline-*d*-riboside and dimethyl xanthosine Levene and Sobotka<sup>4</sup> drew the conclusion that the two substances were identical. Since that time, however, it has been shown that the usual ring structure of the ribose component of the naturally occurring nucleosides was <1,4>, or furanose, whereas the free sugars most easily exist in the <1,5>, or pyranose structure. Thus it would appear that either the synthetic theophylline-*d*-ribose was of the furanose ring structure or that it was not identical with dimethyl xanthosine.

A comparison of the rate of hydrolysis of theophylline-*d*-allomethyloside which undoubtedly contains the <1,5> pyranose ring structure with theophylline-*d*-riboside was made. As can be seen from Table I, theophylline-*d*-allomethyloside is hydrolyzed about 8 times as fast as theophylline-*d*-riboside. It has been previously shown,<sup>5</sup> however, that the rate of hydrolysis of nucle-

<sup>4</sup> Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, **65**, 463 (1925).

<sup>5</sup> Levene, P. A., and Compton, J., *J. Biol. Chem.*, **114**, 9 (1936).

osides can reveal little information in regard to the ring structure of the sugar component. Therefore, no valid conclusions can be drawn from the present work except to state that their rates are of about the same order of magnitude. It would thus appear that the ring structure of the ribose component of synthetic theophylline-*d*-riboside requires further examination and this is now under way.

#### EXPERIMENTAL

*Tetraacetyl-d-Allomethylose*—*d*-Allomethylose (0.3 gm.) was dissolved in 2 cc. of dry pyridine and 1 cc. of acetic anhydride added at 0°. The mixture was allowed to stand overnight at room temperature, after which it was poured with stirring into 100 cc. of ice water. The sirup first separating crystallized upon standing a short time and the product was removed by filtration and thoroughly washed with cold water. After one recrystallization from ethyl alcohol, a melting point of 109–110° was obtained which was unchanged by further recrystallizations. Yield 0.50 gm. The specific rotation of the substance in U.S.P. chloroform was

$$[\alpha]_D^{25} = \frac{+0.42^\circ \times 100}{2 \times 2.012} = +10.4^\circ$$

The composition of the substance agreed with that of a tetraacetyl hexomethylose.

|  |  |
|--|--|
| 5.112 mg. substance:                             | 9.502 mg. CO <sub>2</sub> and 2.835 mg. H <sub>2</sub> O         |
| 6.400 " "  | : 7.688 cc. 0.01 N Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub> |
| C <sub>14</sub> H <sub>20</sub> O <sub>8</sub> . | Calculated. C 50.60, H 6.02, COCH <sub>3</sub> , 51.80           |
| 332.2  | Found. " 50.68, " 6.20, " 51.65                                  |

The substance is soluble in chloroform, benzene, and pyridine, slightly soluble in methyl alcohol and ethyl alcohol, and insoluble in water. When heated for a short time with Fehling's solution, it gave a strong reduction.

*Toluene Addition Compound of Theophylline-Triacetyl-d-Allomethyloside*—1.0 gm. of tetraacetyl-*d*-allomethylose was dissolved in 5 cc. of glacial acetic acid saturated at 0° with dry hydrogen bromide and allowed to stand 1 hour at room temperature. The hydrogen bromide and acetic acid were then removed by



diluting with dry toluene and concentrating under diminished pressure at 40°. This process was repeated until a clear, yellow sirup was obtained which was quite unstable and could not be crystallized. The product was not further characterized but condensation with silver theophylline proceeded smoothly as described below.

Sirupy acetobromoallomethylose (about 1.0 gm.) was dissolved in 75 cc. of dry toluene and 1.0 gm. of silver theophylline added. The mixture was then heated at 95–100° for 4 hours, after which a test portion of the solution contained no halogen. The hot solution was then filtered and allowed to stand overnight in the cold. The small amount of theophylline separating was removed by filtration and the filtrate concentrated to a small volume (about 5 cc.) under diminished pressure at 40°, whereupon the nucleoside began to crystallize. The solution was then heated to boiling, filtered, and the product allowed to crystallize completely in the cold. There was thus obtained 0.65 gm. of material which sintered at 130° and melted to a clear liquid at 140°. The liquid resolidified upon further heating and melted again at 213–214°. The melting point was unchanged after a second recrystallization from toluene. The specific rotation of the substance in methyl alcohol was

$$[\alpha]_D^{25} = \frac{+0.44^\circ \times 100}{2 \times 2.00} = +11.0^\circ$$

The composition of the substance agreed with that of a theophylline triacetyl hexomethylose combined with 1 molecule of toluene of crystallization.

4.606 mg. substance: 9.687 mg. CO<sub>2</sub> and 2.480 mg. H<sub>2</sub>O

6.710 " " : 0.614 cc. N<sub>2</sub> (27° and 753 mm.)

C<sub>19</sub>H<sub>24</sub>O<sub>8</sub>N<sub>4</sub>·C<sub>7</sub>H<sub>8</sub>. Calculated. C 57.33, H 5.91, N 10.29

544.2 Found. " 57.35, " 6.02, " 10.31

The substance is difficultly soluble in cold toluene, which makes its purification very easy. It is soluble in chloroform, benzene, and pyridine; insoluble in petroleum ether and water.

*Theophylline-Triacetyl-d-Allomethyloside*—When the toluene addition compound of theophylline triacetyl allomethyloside is slowly heated to 140° under a high vacuum for 2 hours, the toluene is quantitatively removed. The melting point of the

product thus obtained is 217–218°. The substance, after removal of toluene, may be easily recrystallized from methyl alcohol but the melting point remains unchanged.

The specific rotation of the substance in methyl alcohol was

$$[\alpha]_D^{25} = \frac{+0.50^\circ \times 100}{2 \times 2.004} = +12.5^\circ$$

The composition of the substance agreed with that of a theophylline triacetyl hexomethyloside

4.392 mg. substance: 8.112 mg. CO<sub>2</sub> and 2.050 mg. H<sub>2</sub>O

6.212 " " : 0.686 cc. N<sub>2</sub> (28° and 757 mm.)

C<sub>15</sub>H<sub>24</sub>O<sub>8</sub>N<sub>4</sub>. Calculated. C 50.42, H 5.36, N 12.38

452.2 Found. " 50.36, " 5.22, " 12.48

The substance is easily soluble in hot toluene from which the product crystallizes with 1 molecule of toluene of crystallization. The substance does not reduce boiling Fehling's solution, but after hydrolysis with dilute mineral acids gives a strong reduction.

*Theophylline-d-Allomethyloside*—0.8 gm. of theophylline triacetyl allomethyloside (m.p. 217–218°) was dissolved in 30 cc. of methyl alcohol and 1.5 cc. of *N* barium methylate added. The solution was allowed to stand 20 hours at 15°, after which the barium was precipitated by a stream of carbon dioxide. During this process a small amount of water (5 to 10 cc.) was added to aid in the decomposition of the barium methylate. The solution was then filtered and concentrated to a thick sirup under diminished pressure at 40°. The product was obtained anhydrous by dissolving in absolute ethyl alcohol and concentrating to a thick sirup under diminished pressure. This process was repeated several times, after which the amorphous solid was placed under a high vacuum at 100° for 2 hours. The product was then dissolved in a small volume of hot ethyl alcohol and, upon cooling, crystallization began. There was thus obtained 0.35 gm. of material, m.p. 167–168°. Further recrystallization from ethyl alcohol did not change the melting point. The specific rotation of the substance in water and ethyl alcohol was as follows:

$$[\alpha]_D^{25} = \frac{-0.88^\circ \times 100}{2 \times 2.01} = -21.9^\circ \text{ (water)}$$

$$[\alpha]_D^{25} = \frac{-0.13^\circ \times 100}{1 \times 2.008} = -6.5^\circ \text{ (ethyl alcohol)}$$

The composition of the substance agreed with that of a theophylline hexomethyloside.

4.314 mg. substance: 7.504 mg. CO<sub>2</sub> and 2.170 mg. H<sub>2</sub>O

4.810 " " : 0.759 cc. N<sub>2</sub> (30° and 751.8 mm.)

C<sub>11</sub>H<sub>11</sub>O<sub>6</sub>N<sub>4</sub>. Calculated. C 47.83, H 5.52, N 17.17

326.1 Found. " 47.43, " 5.62, " 17.58

The substance is soluble in water, methyl alcohol, and ethyl alcohol; insoluble in chloroform, ether, toluene, and benzene. It does not reduce boiling Fehling's solution but, after hydrolysis with mineral acids, it gives a strong reduction.

TABLE I

Comparison of Rate of Hydrolysis of Theophylline-*d*-Allomethyloside with Theophylline-*d*-Riboside\* in 0.1 *N* HCl at 100°

$$k = (2.303/t) \log (\alpha_0 - \alpha_f) / (\alpha_i - \alpha_f)$$

| Time     | Theophylline- <i>d</i> -allomethyloside, $[\alpha]_D^{25}$ | Per cent hydrolysis                     |                                  | Theophylline- <i>d</i> -allomethyloside | Theophylline- <i>d</i> -riboside |
|----------|--|---|----------------------------------|---|----------------------------------|
|          |  | Theophylline- <i>d</i> -allomethyloside | Theophylline- <i>d</i> -riboside |   |                                  |
| min.     | degrees  |   |                                  | $k \times 10^{-3}$                      | $k \times 10^{-4}$               |
| 0        | -21.2  | 0                                       | 0                                |   |                                  |
| 60       | -16.1  | 23.5                                    | 11.0                             |   | 7.81                             |
| 120      | -10.5  | 49.4                                    | 17.6                             | 5.66                                    | 7.00                             |
| 180      | -6.8   | 66.3                                    |                                  | 6.05                                    |                                  |
| 240      | -4.7   | 76.0                                    | 31.6                             | 5.87                                    | 6.87                             |
| $\alpha$ | +0.5   | 100.0                                   | 100.0                            |   |                                  |

\*Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, **65**, 463 (1925).

**Reacetylation of Theophylline-*d*-Allomethyloside**—90 mg. of theophylline-*d*-allomethyloside (m.p. 167–168°) were dissolved in 1 cc. of pyridine and 0.5 cc. of acetic anhydride added at 0°. The mixture was allowed to stand at room temperature overnight, after which it was poured into 20 cc. of ice water. After standing for some time in the cold, the solution was thoroughly extracted with chloroform. The chloroform extract was freed of pyridine by washing twice with ice-cold 5 per cent sulfuric acid, twice with saturated sodium bicarbonate solution, and finally twice with water. The solution was then dried over calcium chloride, filtered, and concentrated under diminished pressure

to a thick sirup from which the last traces of chloroform were removed by dissolving in a small volume of toluene and concentrating under diminished pressure. As the concentration proceeded, the product crystallized from solution as the toluene addition compound of theophylline-triacetyl-*d*-allomethyloside. After one recrystallization from toluene, a melting point of 140° was obtained. After heating at 100° for 2 hours under a high vacuum, 60 mg. of substance with a melting point of 217-218° were obtained, unchanged by further recrystallizations. A mixed melting point of this material with the original theophylline-triacetyl-*d*-allomethyloside melted at 217-218°.

*Acid Hydrolysis of Theophylline-d-Allomethyloside*—0.1238 gm. of theophylline-*d*-allomethyloside was dissolved in exactly 5.0 cc. of 0.1 N hydrochloric acid solution and heated at 100° in a glass-stoppered flask. At the end of the specified time the flask was immediately cooled to 0° and the rotation of the solution observed. The flask was then replaced in the water bath. The results obtained in this manner are given in Table I.



## **COLORING MATTERS OF GRIMES GOLDEN, JONATHAN, AND STAYMAN WINESAP APPLES\***

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Red color development in apples is important both to the grower and consumer of this fruit. The apple grower is well aware that color is one of the principal factors affecting market value and keeping quality; well colored fruit almost invariably commands higher prices and is the least susceptible to storage scald. Changes in chemical composition that take place during color development of the fruit while on the tree result in a skin condition that is highly resistant to scald which might occur after the fruit is placed in storage. Furthermore, the maximum development of pigmentation during maturation is a criterion of that particular stage of maturity at which the fruit will attain satisfactory flavor and aroma and also be most edible in other respects.

Owing to the economic value which is placed upon color in apples it is not surprising, therefore, that attempts have been made to study (1-5) the factors which are responsible for and influence color production. However, before we can hope for a more thorough understanding of the complex subject of pigmentation in apples, it is necessary to possess a knowledge of the chemical nature of the pigments involved and of the biochemical

\* Food Research Division Contribution No. 304.

A brief note of the writer's final conclusions appeared on p. 10 of the "Report of the Chief of the Bureau of Chemistry and Soils," United States Department of Agriculture, released August 31, 1935. After the completion of the present investigation, the work of Duncan and Dustman on "The anthocyanin pigment of the Winesap apple" came to the author's attention through its presentation, September 8, at the meeting of the American Chemical Society at Pittsburgh; it has since been published (Duncan, I. J., and Dustman, R. B., *J. Am. Chem. Soc.*, **58**, 1511 (1936)).

reactions responsible for the formation of the red or anthocyanin pigment and its chromogenic precursor.

The object of the present investigation was, therefore, not only to identify more completely the red pigment occurring in Jonathan and Stayman Winesap apples but also to isolate and identify the flavonol glycoside accompanying the red pigment in Jonathan apples and occurring alone in Grimes Golden.

Robinson and Robinson (6) have by qualitative methods tentatively identified the anthocyanin of red apples as a 3-monoside of cyanidin, and in a previous communication the writer has reported the occurrence of quercetin in peels of the McIntosh apple (7).

As a result of the present work, the anthocyanin pigment of Jonathan and Stayman Winesap apples has been identified as idaein, a galactoside of cyanidin. Likewise, the yellow cell sap-soluble pigment occurring in Grimes Golden and Jonathan apples has been identified as a new and previously unreported galactoside of quercetin. It may safely be assumed that these two pigments probably occur in all corresponding varieties of apples.

A fairly complete discussion is reported elsewhere (8, 9) of the possible relationship between flavonol glycosides and anthocyanins. We have in the case of the apple another instance in which the anthocyanin corresponds to the flavonol glycoside in being its reduction product. A similar relation between the pigments of corn husks (9) is the only other case where a correspondence has been shown between the glycoside of an anthocyanidin and of a flavonol.

The evidence obtained thus far favors the possibility of a conversion in apples of the quercetin galactoside or chromogen to red idaein. In red apples the presence of the complete mechanism for such a conversion is postulated, whereas in apples of the Grimes Golden type there is lacking or inhibited some factor or condition other than the chromogenic substance, since the latter is present but not capable of being reduced to its homologous anthocyanin.

#### EXPERIMENTAL

The material used in this investigation consisted of coarsely ground air-dried peels obtained from Grimes Golden, Jonathan, and Stayman Winesap apples. The first two varieties were

harvested the latter part of September and the Winesaps in October. The fruit in each case was of good grade and above average in color. The skins were removed from the fruit with a small commercial apple paring machine and immersed in warm dilute salt water, then pressed by hand and dried with the aid of a fan. The use of salt solution successfully inhibited the characteristic discoloration or browning of freshly cut apple tissue. The ground material was extracted first with petroleum ether (b.p. 30-60°) and then with ethyl ether to remove the wax-like surface constituents.

*Yellow Flavonol Pigment from Grimes Golden and Jonathan Apples*  
*A New Galactoside, 3-Galactosidylquercetin*

*Extraction from Grimes Golden Apples*—40 bushels of this variety, weighing 1827 pounds and consisting of 10,180 apples, yielded 11,290 gm. of air-dried peels. The petroleum ether extract amounted to 367 gm. or 3.3 per cent of the peels and the ether extraction, which followed, removed a further 473 gm. or 4.2 per cent.

The extracted peels were subsequently percolated with cold 90 per cent ethyl alcohol until most of the color was extracted. The alcoholic extract was evaporated to small volume *in vacuo* and the syrupy residue diluted with a little water and placed in an ice box. After several days the gummy precipitate was removed by filtration and the filtrate repeatedly extracted with ether to remove any free flavonol resulting from hydrolysis of its glycoside during drying or subsequent treatment of the peels. After evaporation of the ether, 2.8 gm. of crude quercetin were obtained. This material was purified and identified by means of its acetyl derivative, which had the same melting point, 294-296°, as authentic pentaacetylquercetin. The ether-extracted solution was then exhaustively extracted with acetic ether to remove the glycosidal pigment. Evaporation under slightly reduced pressure of the acetic ether, after drying over anhydrous sodium sulfate, left a yellowish brown residue. This residue was dissolved in the minimum quantity of boiling absolute alcohol and a little hot water added. On cooling the pigment separated in a semi-crystalline condition. It was further purified by successive



crystallizations from boiling absolute alcohol, boiling 1 per cent aqueous pyridine, and boiling water.

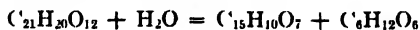
By this method 8.5 gm. of pure air-dried coloring matter were obtained, having a melting point of 236.5–237.5°. Obtained by crystallization from hot water, the substance formed microscopically fine, very dense, aggregates of silky crystals (Fig. 1) which in mass exhibited a primrose-yellow color. Analysis<sup>1</sup> of the air-dried substance gave C 52.31, H 4.79, H<sub>2</sub>O 3.88. C<sub>21</sub>H<sub>20</sub>O<sub>12</sub>·H<sub>2</sub>O



FIG. 1 3-Galactosidylquercetin

requires C 52.26, H 4.59, H<sub>2</sub>O 3.73. After drying at 125°, the values were C 54.18, H 4.47. C<sub>21</sub>H<sub>20</sub>O<sub>12</sub> required C 54.29, H 4.34.

*Hydrolysis of Galactoside*—Upon hydrolysis, the yellow glycoside gave quercetin and galactose in molecular proportions which are in accord with those calculated from the equation



The anhydrous material, 0.9507 gm., was dissolved in 75 ml. of hot water and 25 ml. of water containing 2 ml. of concentrated

<sup>1</sup> The writer is greatly indebted to Dr. R. T. Milner and Mrs. Mildred S. Sherman, Fertilizer Investigations, Bureau of Chemistry and Soils, for analytical results reported.

sulfuric acid were added. The solution was then boiled until hydrolysis was complete and set aside overnight in an ice box. Crystals of the aglycone separated from the solution during boiling and cooling. These were collected in a Gooch crucible and washed with cold water. Recovery of anhydrous quercetin amounted to 64.87 per cent.  $C_{21}H_{30}O_{12}$  requires 65.08 per cent of quercetin,  $C_{18}H_{16}O_7$ .

The filtrate from the hydrolysis mixture was freed of sulfuric acid with barium carbonate and concentrated *in vacuo*. After adding methyl phenylhydrazine and acetic acid and subsequently sufficient alcohol for solution, the mixture was heated on the steam bath. *d*-Galactose methyl phenylhydrazone was formed which after recrystallization melted at 189–190°. Methyl phenylhydrazone prepared from pure galactose of authentic origin melted at 190°. Both samples yielded identical optical and crystallographic data,<sup>2</sup> which are here summarized. In parallel polarized light (crossed nicols) the substance, consisting of colorless rods and needles, shows straight extinction and negative elongation. In convergent polarized light (crossed nicols) the biaxial interference figure shows the acute bisectrix and a very small axial angle. The refractive indices as determined by the immersion method are  $\alpha = 1.565$  (very common lengthwise on rods),  $\beta =$  not determined,  $\gamma = > 1.734$  (methylene iodide); both  $\pm 0.003$ .

Quercetin, obtained from the galactoside by hydrolysis, was identified by conversion into its acetyl derivative and by combustion. It was acetylated by boiling 1 hour with acetic anhydride and anhydrous sodium acetate. The product, obtained by crystallization from 95 per cent alcohol consisted of colorless rods melting at 194–196°. On analysis the dried substance gave C 58.58, H 4.16. Pentaacetylquercetin,  $C_{18}H_{16}O_7(C_2H_3O)_5$ , requires C 58.57, H 3.93. Hydrolysis of the acetyl derivative yielded theoretical quantities of quercetin, 0.6224 gm. of substance yielding 0.3667 gm. of the hydrolyzed product, which corresponded to 58.91 per cent of quercetin. Theory requires 58.98 per cent of quercetin. The compound obtained responded to all the well known tests for quercetin and gave further confirmatory

<sup>2</sup> All optical and crystallographic examinations were kindly made by Mr. G. L. Keenan, of the Food and Drug Administration, United States Department of Agriculture.

results on analysis, C 59.52, H 3.39. Quercetin,  $C_{15}H_{10}O_7$ , requires C 59.59, H 3.34.

*Position of Sugar in Galactosidylquercetin*—The position of the sugar was determined by obtaining 3-hydroxy-5,7,3',4'-tetramethoxyflavone on hydrolyzing the methylated quercetin galactoside. The anhydrous galactoside was suspended in dry ether and treated with successive quantities of diazomethane over a long period of time. When methylation was complete the residue obtained on evaporation of the ether was crystallized from absolute alcohol and hydrolyzed on the steam bath with 0.5 per cent sulfuric acid. The insoluble residue so obtained was crystallized from dilute alcohol; it appeared as slightly yellow needles melting at 194.5–195° and remelting at 195–196°. No depression in the melting point occurred when this material was admixed with authentic 3-hydroxy-5,7,3',4'-tetramethoxyflavone prepared from pure isoquercitrin according to the method of Nakamura, Ohta, and Hukuti (10). Analysis of the dried substance prepared from galactosidylquercetin gave C 63.61, H 5.47.  $C_{15}H_{10}O_2(OH)(OCH_3)_4$  requires C 63.66, H 5.07.

From the above results it is evident, therefore, that the flavonol galactoside occurring in Grimes Golden apples is identified as 3-galactosidylquercetin.

*Extraction from Jonathan Apples*—20 bushels of well colored Jonathan fruit, weighing 884 pounds and totaling 3120 apples, yielded 4395 gm. of air-dried peels. The petroleum ether extract amounted to 120 gm. or 2.7 per cent of the peels and the subsequent ether extraction removed 280 gm. or 6.3 per cent.

For the extraction of the coloring matter the same method was employed as described in detail under the preparation from Grimes Golden apples. The total pure air-dried substance so obtained amounted to 3.5 gm. In addition 0.5 gm. of crude non-glycosidal pigment was recovered. The glycoside was identical in appearance and behavior with 3-galactosidylquercetin. Further confirmation was secured by determining the optical crystallographic properties of the glycoside obtained from both sources, which proved to be the same in each case. They are here summarized. In ordinary light, the compound is primrose-yellow in color and consists of rods, which when separated and broken up for optical study are almost colorless. In parallel polarized light, the extinc-

tion is straight and the sign of elongation negative. The double refraction is extremely strong ( $\gamma - \alpha = > 0.117$ ). In convergent polarized light, interference figures are rarely seen. The refractive indices are:  $\alpha = 1.620$  (shown lengthwise on rods, but not common),  $\beta =$  not determined,  $\gamma = > 1.737$ ; an intermediate index,  $n_i = 1.660$ , is shown lengthwise on rods and is common; all  $\pm 0.003$ .

*Red Anthocyanin Pigment from Jonathan and Stayman Winesap Apples*

*Idaein or 3- $\beta$ -Galactosidylcyanidin*

*Extraction from Jonathan Apples*—46 bushels, weighing 1943 pounds and containing 9160 apples, yielded 10,093 gm. of air-dried peels. The petroleum ether extract amounted to 445 gm. or 4.4 per cent of the peels and the subsequent ether extract amounted to 624.4 gm. or 6.2 per cent.

The petroleum ether- and ether-extracted peels were then percolated with cold 0.5 per cent methyl-alcoholic hydrogen chloride until most of the red color was removed. The extract, amounting to 36 liters, was evaporated under reduced pressure to approximately 7 liters and then treated with 3 volumes of ether. The bulk of the pigment separated as a bright red gummy residue, which was dissolved in a large volume of 0.01 per cent aqueous hydrochloric acid. After filtering, the solution was fractionally treated with neutral lead acetate which precipitated the pigment as the lead salt. The middle fraction, which was deep blue in color, contained most of the anthocyanin pigment and a quantity of 3-galactosidylquercetin. Excess of lead acetate yielded a third greenish fraction which contained relatively little anthocyanin but a larger quantity of the flavonol glycoside.

The deep blue lead salt was finely ground after drying and decomposed in cold water with hydrogen sulfide, the lead sulfide filtered off, and the red liquid shaken out with ethyl acetate to remove the yellow pigment. This extract, together with that obtained from the third lead precipitate, yielded 3.4 gm. of pure 3-galactosidylquercetin and 1 gm. of crude quercetin. The acetic ether-extracted liquid containing the anthocyanin was treated with neutral lead acetate to recover the pigment in the form of its

blue salt. After drying and grinding, it was decomposed with 10 per cent methyl alcoholic hydrogen chloride, and the lead chloride removed by filtration through talc with suction. Addition of ether to the filtrate precipitated the pigment as a deep red gummy material. After air drying, the substance was converted into the picrate.

*Purification by Means of Picrate*—The crude pigment was pulverized in an agate mortar and dissolved in hot (60–70°) saturated aqueous picric acid. Insoluble gummy impurities were filtered off while hot, and the deep orange-red solution was kept in a cool place for several days. The crude picrate which separated was recrystallized from half saturated picric acid solution. As thus obtained, it formed glistening red-brown rods. The picrate was decomposed, 10 per cent methyl alcoholic hydrogen chloride being employed, and the parent substance recovered by precipitation with ether. The pigment was washed with ether to remove excess picric acid and then dried in a desiccator and ground. It was then dissolved in 8 ml. of hot 0.01 per cent aqueous hydrochloric acid and 5 volumes of warm 3 per cent ethyl alcoholic hydrogen chloride were added. The anthocyanin slowly separated and a larger yield was obtained by permitting the liquid to evaporate slowly in a shallow dish loosely covered with filter paper. The yield of pigment amounted to 1.2 gm. Since the pigment was still too impure to crystallize, it was dissolved in 125 ml. of hot methyl alcohol, filtered, and 18 ml. of 20 per cent hydrochloric acid were added. After filtration, the mixture was allowed to evaporate slowly and the substance which separated was dissolved in 12 ml. of cold water and 8.4 ml. of 10 per cent methyl alcoholic hydrogen chloride were added. Overnight in a shallow dish covered with filter paper, the substance separated in crystalline condition. It was finally recrystallized by solution in 15 ml. of 0.01 per cent aqueous hydrochloric acid, filtration, and the addition of 2 ml. of concentrated hydrochloric acid and 10 ml. of a mixture of ethyl and methyl alcohols. It was placed in a shallow dish overnight; separation was practically complete, with the mother liquor only moderately colored. The deposit of coloring matter had a glistening bronze-colored appearance and consisted of dense crystalline aggregates of leaflets. After washing with 7 per cent hydrochloric acid and air drying,

the material weighed 0.65 gm. The filtered material, while still moist, had a reddish brown color, but after drying it then exhibited a green reflex.

*Idaein Chloride*,  $C_{21}H_{21}O_{11}Cl$ —The coloring matter, as obtained above, contained  $2\frac{1}{2}$  molecules of water of crystallization. A second sample, crystallized under slightly different conditions which have not been carefully studied, yielded 1 instead of  $2\frac{1}{2}$  molecules. The substance was analyzed, both in its air-dried state and after drying at  $100^\circ$  in a very high vacuum obtained by means of the mercury vapor pump. The air-dried material gave C 47.46, H 4.68,  $H_2O$  9.11. Idaein chloride,  $C_{21}H_{21}O_{11}Cl \cdot 2\frac{1}{2}H_2O$ , requires C 47.57, H 4.94,  $H_2O$  8.51. The second sample, referred to above, gave  $H_2O$  3.66.  $C_{21}H_{21}O_{11}Cl \cdot 1H_2O$  requires  $H_2O$  3.58. The anhydrous material gave C 52.34, H 4.89, Cl 7.25.  $C_{21}H_{21}O_{11}Cl$  requires C 52.00, H 4.37, Cl 7.32.

In appearance and behavior with certain reagents the pigment agreed in every respect with idaein chloride isolated from the mountain cranberry by Willstätter and Mallison (11) and synthesized by Grove and Robinson (12). It is soluble in water, forming a dark reddish brown color which on dilution assumes an orange cast. It is almost as easily soluble in 0.01 per cent aqueous hydrochloric acid, fairly readily soluble in 0.5 per cent acid, but in general becomes less soluble as the concentration of acid is increased. It is almost insoluble in 7 per cent hydrochloric acid. Sodium carbonate imparts a violet color to its aqueous solution, whereas sodium hydroxide gives a blue color which becomes green and finally yellow. In alcohol, ferric chloride yields a blue color, which changes to violet in dilution with water.

*Hydrolysis of Glycoside*—Idaein chloride, 0.9372 gm., was dissolved in 12 ml. of boiling water, and 10 ml. of concentrated hydrochloric acid were added. The solution was then boiled for 3 minutes, cooled immediately, and set aside overnight in an ice box. Crystals of the aglycone began to separate from the solution during boiling and cooling. These were collected, washed with 7 per cent hydrochloric acid, and air-dried. Recovery of air-dried cyanidin chloride amounted to 70.6 per cent.  $C_{21}H_{21}O_{11}Cl$  yields 70.27 per cent of cyanidin chloride,  $C_{15}H_{11}O_6Cl \cdot 1H_2O$ .

The slightly red filtrate from the hydrolysis mixture was shaken

with amyl alcohol to remove traces of cyanidin chloride, then neutralized with lead carbonate. The lead chloride was filtered off and the excess lead removed by means of hydrogen sulfide. The solution was concentrated *in vacuo* and the sugar converted into its methyl phenylhydrazone by heating with methyl phenylhydrazine and acetic acid, after adding sufficient alcohol to insure solution. The precipitated *d*-galactose methyl phenylhydrazone was recrystallized, after which it melted at 186–186.5°. Although this melting point is slightly lower than that reported for the pure substance, no doubt existed as to its identity, since it agreed in optical crystallographic properties with those obtained for the corresponding derivative obtained from 3-galactosidylquercetin and from the same derivative prepared from authentic galactose.

*Cyanidin Chloride*,  $C_{15}H_{11}O_6Cl$ —The hydrolytic product of idaein crystallized from hot 20 per cent hydrochloric acid as long, slender brown-red needles which under the microscope appeared to be perfectly homogeneous and free of amorphous particles. The sample, after drying at 111° in a high vacuum, gave C 55.93, H 3.57, Cl 10.84.  $C_{15}H_{11}O_6Cl$  requires C 55.80, H 3.44, Cl 10.99. The product agreed in appearance and behavior with authentic cyanidin chloride prepared from chrysanthemin chloride (9).

*Extraction from Stayman Winesap Apples*—31 bushels of mature and unusually well colored Stayman Winesap apples, weighing 1470 pounds and containing 4780 apples, yielded 8252 gm. of air-dried peels. The ether extract amounted to 685 gm. or 8.3 per cent.

To isolate the pigment from this material a much shorter method was used than that employed in the case of Jonathan fruit. The ether-extracted peels were percolated with 0.25 per cent methyl alcoholic hydrogen chloride until 17 liters of extract were obtained. This extract was concentrated *in vacuo* to approximately 3 liters and then 3 volumes of ether were added. The dark gummy precipitate was washed with ether and dissolved in the minimum quantity of water, then filtered and treated with neutral lead acetate solution as long as the blue lead salt was formed. The lead salt was collected, washed, and dried, after which it was finely ground and decomposed with 10 per cent methyl alcoholic hydrogen chloride. The deep red-colored liquid, free of lead chloride, was treated with 5 volumes of ether.

The crude pigment which separated was converted into the picrate which was purified by recrystallization from half saturated aqueous picric acid. Approximately 4 gm. of air-dried picrate were thus obtained.

The parent substance was regenerated and recovered from the picrate with 10 per cent methyl alcoholic hydrogen chloride and precipitation with ether. The air-dried coloring matter weighed 2.75 gm. It was further purified by recrystallization from hydrochloric acid which was diluted with alcohol. 0.5 gm. of pigment was dissolved in 50 ml. of water and 10 ml. of concentrated hydrochloric acid were added, and finally after filtration 20 ml. of absolute alcohol were added and the mixture was allowed to evaporate in a shallow dish covered with filter paper. The crystalline pigment began to separate within  $\frac{1}{2}$  hour and the precipitation was practically complete overnight. After several recrystallizations the coloring matter was obtained as dense clumps of leaflets which exhibited a green reflex. The total yield of air-dried substance amounted to 2 gm. Analysis of the anhydrous substance gave C 52.03, H 4.44, Cl 7.60. Idaein chloride,  $C_{21}H_{21}O_{11}Cl$ , requires C 52.00, H 4.37, Cl 7.32.

In appearance and behavior it agreed in every respect with idaein chloride obtained from Jonathan apples and likewise yielded cyanidin chloride and galactose upon hydrolysis.

#### SUMMARY

As a result of the present work, the flavonol glycoside occurring in the skins of Grimes Golden and Jonathan apples and the anthocyanin coloring matter present in Jonathan and Stayman Winesap apples have been identified as 3-galactosidylquercetin and 3- $\beta$ -galactosidylcyanidin, respectively. It may safely be assumed that the flavonol glycoside occurs in all varieties of apples and that 3- $\beta$ -galactosidylcyanidin, or idaein, occurs in all red varieties.

Both coloring matters yield on hydrolysis molecular proportions of aglycone (quercetin and cyanidin, respectively) and galactose.

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A crystalline protein, possessing the properties of tobacco mosaic virus, has been prepared from the juice of Turkish tobacco plants infected with this virus (1). The fact that the protein carries all the virus activity and that no way has been found of obtaining this activity separate from the protein gives especial interest to studies of its physical properties. The present paper is a record of an investigation of the rates at which virus proteins, obtained from several different plant hosts diseased with distinct strains of tobacco mosaic virus, sediment in an intense gravitational field.<sup>1</sup> These sedimentation rates, as shown by the experiments of Svedberg (3), are quantities which depend upon the weight and the shape of molecules in solution.

<sup>1</sup> The sedimentation and electrophoretic behaviors of virus protein obtained from Turkish tobacco plants diseased with the ordinary strain of tobacco mosaic virus have been described by Eriksson-Quensel and Svedberg (2). The results upon similar material, as recorded in these two investigations, are in substantial agreement.

virus, on the other hand, produces a systemic infection in Turkish tobacco and *Nicotiana sylvestris* and symptoms so mild that the inexperienced observer cannot distinguish between a healthy and a diseased plant. Very dilute solutions of the crystalline proteins prepared from plants infected with each of these strains produce on inoculation to healthy plants the same type of symptoms that were manifested by the diseased plants from which they were extracted. In other words, infection with aucuba protein results in aucuba mosaic disease and infection with the "masked" protein results in the "masked" disease. Grant (5) demonstrated that ordinary tobacco mosaic virus can multiply in over twenty-nine different species of plants representing fourteen widely separated families. Tobacco mosaic virus protein isolated from infected tomato plants, a plant species fairly closely related to tobacco and in the same family, was found (6) to be indistinguishable from that previously isolated from mosaic-diseased Turkish tobacco plants. Recently the same or a closely related virus protein was isolated from infected phlox plants (unpublished experiments of one of us, W. M. S.), a plant species belonging to a family widely separated from that to which tomato and tobacco belong. Such crystalline proteins, isolated from different families of plants and corresponding to strains capable of producing different biological reactions, were subjected to an ultracentrifugal analysis in order to determine whether or not they would show differences in sedimentation rates.

The ultracentrifuge used in these studies was of the simple air turbine type already described (7). Sedimentation constants were determined with it by use of the classical absorption and refractive index methods developed in the laboratory of Svedberg (3). The experimental arrangement for absorption studies with the air-driven ultracentrifuge has been outlined; a similar description of the equipment employed for the refractive index observations will be published shortly. For the present experiments the optical systems and cameras were so placed that photographs by the two methods could be made simultaneously on the same sedimenting sample.

The immediately striking result of the ultracentrifugal examination of solutions of mosaic virus proteins is their very large sedimentation constants. Of the order of  $s = 200 \times 10^{-13}$  cm. sec.<sup>-1</sup>

dynes<sup>-1</sup>, these constants are the largest yet found for any protein; they refer to molecules with molecular weights probably in excess of 10,000,000. In contrast to this, the proteins of healthy plants, prepared by the same procedure used in extracting the virus proteins, do not contain a demonstrable amount of molecules with weights as great as 30,000; there is no observable sedimentation from a solution of normal plant protein maintained in a field of 180,000 times gravity for 90 minutes.

The question might be raised as to whether the molecules of the purified protein are the same as those present in the plant juice itself. To answer this, an absorption run was made on juice from tobacco plants infected with the ordinary strain of virus. Though this juice contained much ultraviolet-absorbing material associated with molecules too small to sediment in the gravitational fields used in the present experiments, the boundary due to the sedimentation of the heavy virus protein was clearly measurable. In another experiment several cc. of the juice were centrifuged for 3 hours at 25,000 R.P.M., the field being such that all the heavy protein was thrown down to form a fibrous crystalline mass (8) at the bottom of the centrifuge tube. A solution of this mass in saline, when run in the analytical ultracentrifuge, gave the same sedimentation constant as that found for protein in the original juice and for solutions of the protein purified and crystallized in the usual fashion (9).

The first measurements on samples of the ordinary tobacco mosaic and the aucuba mosaic proteins showed that, while they yield similar values for  $s$ , there are clearly marked differences. We have accordingly analyzed solutions of crystallized virus proteins from a wide variety of sources—from two or more specimens of the ordinary, aucuba, and “masked” strains of tobacco mosaic virus, from the ordinary strain grown in tomato and phlox as well as in Turkish tobacco plants, and from this strain taken from plants of various ages, and also from a so called “purified” strain developed from a single lesion. Examinations have also been carried out of the proteins inactivated by several chemical reagents. Solutions have been centrifuged at pH values ranging from about 2.2 to 11.4 and at concentrations between 0.5 and 2.5 mg. of protein per cc.; for a cell 2 mm. thick the optimum concentration for absorption measurements is about 1.5 mg. of protein per cc. These results are collected in Table I.

TABLE I  
Sedimentation Constants of Virus Proteins

| Virus           | Host    | pH      | Sedimentation constant $s_{20}^0$ | Remarks  |
|-----------------|---------|---------|-----------------------------------|--|
| Tobacco, August | Tobacco | 7.0     | 190                               | Untreated plant juice  |
| "               | "       | 7.0     | 187                               | Solution of ppt. obtained by centrifuging juice  |
| " March         | "       | 7.0     | 186                               | No sedimentable molecules<br>Much heavy colloidal material   |
| " June          | "       | 7.0     | 188                               |  |
| " March         | "       | 8.3     | 176                               |  |
| " June          | "       | 9.3     | 173                               |  |
| " July          | "       | 11.4    |                                   | Runs made on 2 mo.-old solutions<br>Mostly heavy colloidal material, impossible to photometer<br>" "<br>Different sample from preceding; contained unsedimentable material |
| Aucuba, March   | "       | 7.0     | 253                               |  |
| " "             | "       | 8.3     | 244                               |  |
| " May           | "       | 8.3     | 242                               |  |
| " "             | "       | 7.0     | 212                               |  |
| " "             | "       | 7.0     | 224                               |  |
| " "             | "       | 7.0     | 223                               |  |
| " July          | "       | 6.1     | 260 Ca.                           |  |
| " "             | "       | 2.2 Ca. | 240 Ca.                           |  |
| Masked, March   | "       | 7.0     | 215                               |  |
| " May           | "       | 7.0     | 218                               | Proteins prepared from tobacco and tomato plants of same age, inoculated at same time with same virus sample   |
| " "             | "       | 7.0     | 211                               |  |
| Tobacco, April  | Tomato  | 7.0     | 207                               |  |
| " May           | "       | 7.0     | 207                               |  |
| " August        | Tobacco | 7.0     | 173                               |  |
| " "             | Tomato  | 7.0     | 173                               |  |
| " "             | Phlox   | 7.0     | 174                               |  |

| <i>Chemically Altered Ordinary Tobacco Mosaic Virus Proteins</i> |                        |  |  |
|--|------------------------|--|--|
| Reagent  | Sedimentation constant | Remarks  |  |
| H <sub>2</sub> O <sub>2</sub> , June sample                      | 202                    | Fairly sharp boundary                                    |  |
| " August sample  | 182                    | "  |  |
| HCHO   | 191                    | Considerable molecular heterogeneity                     |  |
| HNO <sub>3</sub>   | 172 Ca.                | Very diffuse boundary allowing only visual estimate of s |  |

| Tobacco, single lesion, April                          | Tobacco, young              |     |             |   |
|--|-----------------------------|-----|-------------|---|
| "  | "                           | 7.0 | 234         | Different sample  |
| "  | "                           | 7.0 | 228         |   |
| "  | "                           | 7.0 | 228         |   |
| "  | "                           | 7.0 | 238         |   |
| Tobacco from young plants, probably single lesion, May | 2 wks. after inoculation    |     |             |   |
| "  | Tobacco 3 wks.              | 7.0 | 204         |   |
| "  | " 4 "                       | 7.0 | 195         | Especially diffuse boundary                                     |
| "  | " 5 "                       | 7.0 | 194         | "   |
| "  | " 6 "                       | 7.0 | 212         | "   |
| Tobacco, single lesion, July                           | " 18 days after inoculation | 7.0 | 228         | Some unsedimentable and much heavy colloidal material           |
| "  | Tobacco 35 days             | 7.0 | 179         |   |
| "  | " 42 "                      | 7.0 | 173         |   |
| "  | " 53 "                      | 7.0 | 191         |   |
| Tobacco, July  | " 1 wk. after inoculation   | 7.0 | 203 Average | Double boundary, dilute solution, much un-sedimentable material |
| "  | Tobacco 2 wks.              | 7.0 | 207 Average | Double boundary   |
| "  | " 3 "                       | 7.0 | 184, 217    | Two components of double boundary                               |
| "  | " 5 "                       | 7.0 | 214         | Dilute solution, un-sedimentable material                       |
| "  | " 8 "                       | 7.0 | 184         | Single boundary   |

A few sedimentations were made at rotational speeds up to 300 revolutions per second, but the analyzed results all refer to runs carried out at about 150 R.P.S. At this speed it is convenient to make measurements on photographs taken at 5 minute intervals. Each recorded value is an average drawn from a series of 12 to 13 such exposures. The total change in speed throughout an entire

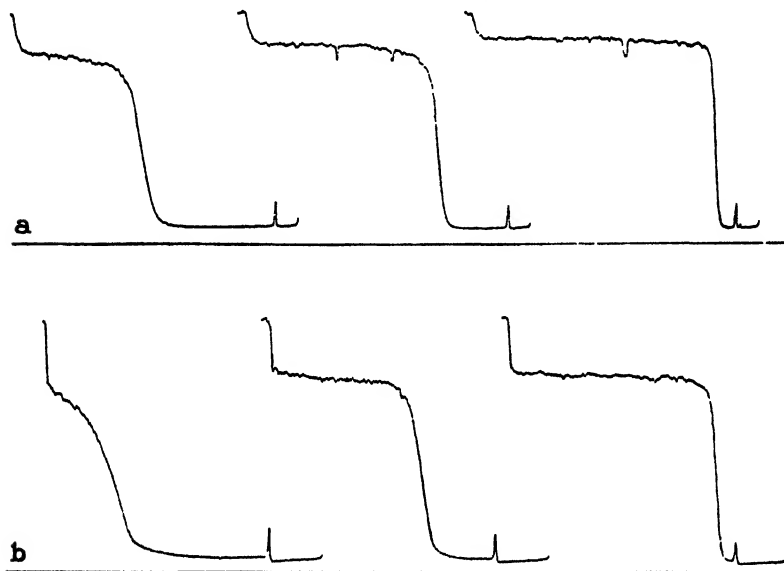


FIG. 1, a. Photometer curves of the first, sixth, and thirteenth absorption photographs of a solution of a single lesion tobacco mosaic protein. This sample showed a fairly high degree of molecular homogeneity. The curve for the first photograph is at the right.

FIG. 1, b. Similar curves from photographs of a solution of a virus protein taken from 3 week-old tobacco plants. This sample exhibited marked molecular heterogeneity.

run never exceeded 3 R.P.S.; over a single 5 minute interval it did not vary by more than 1 R.P.S. The gravitational field at the center of the cell, which was 6.51 cm. distant from the center of rotation, is about 6000 times gravity at 150 R.P.S.

Each absorption photograph was standardized by a series of accurately timed exposures ranging from 5 seconds to 5 minutes. The exposure for the sedimentation pictures was usually 2 seconds;

the longer standardizations were made by interposing a rotating sector. Each photograph was photometered with a recording microphotometer and the curves thus obtained analyzed in the usual fashion for position of sedimenting boundary. Typical photometer curves are reproduced in Fig. 1; tracings of the curves for one run, reduced to concentration distribution throughout the cell, are given in Fig. 2. Calculations from them which illustrate

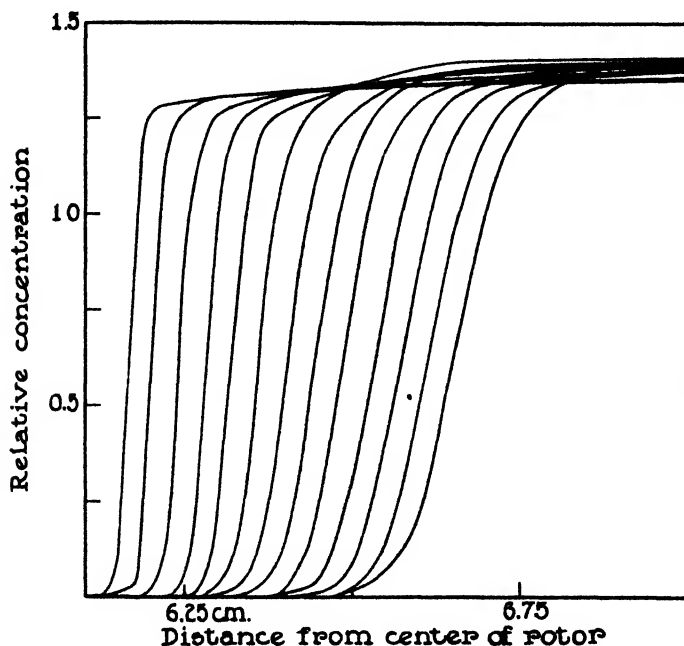


FIG. 2. A family of concentration distribution curves from one run, as calculated from photometer curves such as those of Fig. 1. These curves are reproduced as typical of those given by the tobacco mosaic proteins.

the degree of accuracy of these measurements are shown in Table II. Refractive index runs were made with two independent settings of the optical system. In the case of the molecularly more homogeneous preparations the average sedimentation constants resulting from absorption and refractive index determinations on the same sample agree to within 1 to 2 per cent.

The virus protein samples differed among themselves both in



the rate at which their molecules sedimented and in the homogeneity of the molecules they contained. Some preparations were molecularly homogeneous, as evidenced by their sharp boundaries in absorption photographs (Figs. 1 and 4) and by the absence of abnormal broadening of refractive index peaks as sedimentation proceeded (Fig. 3, a). Other proteins, especially those which during their isolation and purification had been subjected to

TABLE II

*Sedimentation Constants Determined in Typical Absorption Run*

58 day single lesion strain; time interval between exposures = 300 seconds; distance between meniscus and center of rotor = 6.09 cm.; rotor temperature = 27°;  $\Delta x$  = change in position of boundary in interval between exposures;  $n$  = speed in revolutions per second;  $s$  = sedimentation constant.

| Exposure No. | Meniscus-to-boundary | $\Delta x$ | $n$ | $s_{20}^{\circ}$  |
|--------------|----------------------|------------|-----|-------------------|
|              | cm.                  | cm.        |     | $\times 10^{-13}$ |
| 1            | 0.078                |            | 155 |                   |
| 2            | 0.116                | 0.038      | 155 | 214               |
| 3            | 0.157                | 0.041      | 155 | 230               |
| 4            | 0.198                | 0.041      | 155 | 228               |
| 5            | 0.239                | 0.041      | 155 | 227               |
| 6            | 0.280                | 0.041      | 155 | 225               |
| 7            | 0.319                | 0.039      | 155 | 213               |
| 8            | 0.362                | 0.043      | 155 | 233               |
| 9            | 0.404                | 0.042      | 155 | 227               |
| 10           | 0.448                | 0.044      | 155 | 236               |
| 11           | 0.490                | 0.042      | 155 | 224               |
| 12           | 0.531                | 0.041      | 156 | 217               |
| 13           | 0.573                | 0.042      | 156 | 221               |
| Average..... |                      |            |     | 225               |

$$s_{20}^{\circ} = 0.847 \times 225 \times 10^{-13} = 191 \times 10^{-13}.$$

prolonged treatment and to considerable variations in hydrogen ion concentration, showed pronounced molecular heterogeneity with diffuse sedimenting boundaries (Figs. 1 and 5) and peaks that broadened rapidly and died away after the first few pictures (Fig. 3, b). Still other samples contained two molecular species sedimenting at appreciably different rates (Fig. 6).

Partly because the degree of molecular homogeneity of a sample

seemed to depend on the pH at which it was centrifuged, ordinary and aucuba proteins were studied at several pH values. Our results indicate that there is no change in the sedimentation constants of the proteins of the ordinary and aucuba strains between about pH 6 and 9.3. When solutions as acid as pH 5 were cen-

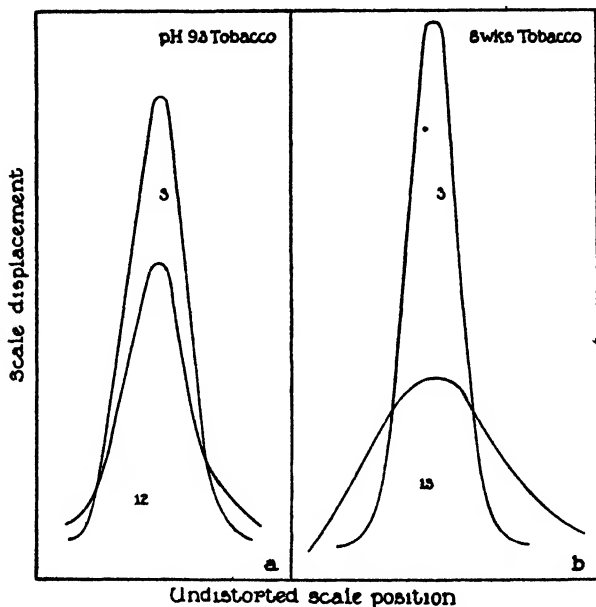


FIG. 3, a. Plots of scale distortion against cell position for peaks in the third and twelfth exposures of a refractive index run on a solution of a molecularly homogeneous protein sample. The two peaks have been plotted with coincident centers to show that there is no abnormal broadening as sedimentation proceeds.

FIG. 3, b. A similar pair of refractive index curves (from the third and thirteenth photographs) from a run on a sample of less molecular homogeneity. The later curve is much broadened.

trifuged, it was found that too little of the protein had remained in true solution to yield an accurate photometer record with a cell 8 mm. thick. Nearly all the ultraviolet-absorbing material sedimented with a very diffuse boundary and a value of  $s$  which was usually of the order of magnitude of 500. It is difficult to escape

the conclusion that this boundary is due to colloidal particles consisting of aggregates of a very few of these large protein molecules. One aucuba sample was put in solution on the acid side of the isoelectric point. Under these circumstances the protein denatures rather rapidly and passes from true solution, but that remaining in solution at about pH 2.2 during a run had the same sedimentation constant that was found for protein in the neutral solution. It is thus apparent that there is neither polymerization nor dissociation of the virus protein molecules in true solution in the range between about pH 2.2 and 9.3. Extreme alkalinity completely disintegrates these large molecules. Thus a solution of the ordinary strain of virus protein run after standing for 10 hours at pH 11.4 contained no demonstrable protein molecules with a weight as great as 30,000. The alkaline disintegration of the large molecule is accompanied by destruction of virus activity, by loss of opalescent appearance in solution, and by the formation of denatured protein.

At the concentration of 1.6 mg. of protein per cc., the molecules of crystalline virus protein centrifuge with constants between 170 and 250 and with no measurable unsedimentable protein in the supernatant layer. This can be seen in Fig. 4 by the fact that the solution above the sedimentation boundary (*b*) is as transparent as the air bubble (*a*) left in the cell. Solutions which had been diluted to 0.5 mg. of protein per cc., however, showed considerable amounts of unsedimentable material (in Fig. 5, *a'* is much lighter than *b'*). Our experiments do not indicate whether the molecular dissociation which therefore occurred on diluting these samples takes place with every virus protein, nor do they show whether it is a reversible phenomenon. No such dissociation in stronger solutions was observed, even after they had stood for several months.

A few of the virus proteins proved to have a high degree of molecular homogeneity, though most of them gave a diffuseness of boundary indicative of some heterogeneity. After double boundaries had been observed in certain samples, it was thought that perhaps this diffuseness might be due to the presence of several unresolved components possessing nearly equal sedimentation rates. Runs were therefore made at twice the rotational speed, and 4 times the gravitational field, but the diffuse

boundaries remained unresolved. Although solutions of physically or chemically inactivated virus proteins have almost always yielded diffuse boundaries, it has not been shown that the degree of polydispersity is a measure of loss of biological activity. Since many different strains of tobacco mosaic virus are known to exist, it appears possible that a given sample of virus protein may consist,



FIG. 4. A print of the series of photographs constituting an absorption run on a solution of the ordinary tobacco virus protein at pH 9.3. The continued sharpness of boundary down to the last picture is indicative of molecular homogeneity. *a*, air bubble; *b*, solution above sedimentation boundary.

FIG. 5. A similar run on a less homogeneous protein sample. Boundaries in the later photographs are diffuse. The presence of unsedimentable material is shown by the greater transparency of the air bubble (*a'*) than of the solution (*b'*) above the sedimenting boundary.

FIG. 6. Photographs of the absorption run on the July, ordinary tobacco virus protein taken from 2 week-old plants. The double boundary is clearly evident in the later pictures.

not of identical molecules, but of a group of closely related molecules which differ slightly in the rate at which they sediment. This factor, as well as the changes caused by chemical treatment, may account for certain of the diffuse boundaries that have been observed.

All samples of aucuba virus protein from old plants have sedi-

mented more rapidly than corresponding samples of the ordinary virus strain. At first it was supposed that a particular strain could be characterized by the sedimentation constant of its protein. This became less probable when a second series of aucuba proteins, prepared 2 months after the first, gave sedimentation constants around 220 instead of 240. In the same way different specimens of the ordinary virus strain have yielded values between about 175 and 195.

The results just described were obtained on proteins isolated from mature plants. A sample of protein obtained from young plants which had been inoculated as early as possible by Dr. W. C. Price with a purified or single lesion strain of virus, derived by Dr. J. H. Jensen from ordinary tobacco mosaic virus, proved to have a high constant of about 230. In order to determine whether this larger constant was due to the new, purified strain of virus or to the isolation of the protein from young plants, an experiment was made in which plants were inoculated with this virus from young plants, and samples of protein were prepared from harvestings made at weekly intervals. Sedimentation rates for most of the series were higher than for previous ordinary virus proteins, though the apparently erratic variation from week to week is noteworthy. It is not known whether this variation is due to actual differences in the protein as it exists in the plants or to differences arising during the isolation and purification of the protein. In a similar experiment made a month later, very young plants were inoculated with the ordinary virus and protein was prepared at weekly intervals for centrifuging. The protein from 1 week-old plants showed a double boundary corresponding to components with  $s$  = about 185 and  $s$  = about 215. These two components continued present for several weeks, though in the protein from the oldest, 8 weeks, plants, only the lighter could be seen. In view of this evidence that protein taken from plants infected with the ordinary strain of virus may consist of two separate molecular species, the single lesion strain of virus was used to inoculate young Turkish tobacco plants and the virus protein was isolated from groups of the plants at intervals up to and including 58 days after inoculation. These samples of virus protein, which were prepared by Dr. H. S. Loring, were found to give no double boundaries, even in the case of the 58 day samples, but as found previously the

different samples gave different constants. The results indicate that the single lesion strain of virus isolated by Dr. Jensen consists of heavier molecules than the bulk of those of the ordinary strain from which it was derived and that the separation of such a strain may tend to increase the molecular homogeneity of the virus protein.

Tobacco mosaic virus protein, isolated from mosaic-diseased tomato plants, was found to have a sedimentation constant in the same range as those of the proteins isolated from Turkish tobacco plants. When the same virus strain was used for inoculation and the protein was harvested from tobacco and tomato plants of the same age, identical sedimentation constants were observed. These samples of protein from tobacco and tomato plants were prepared by Dr. H. S. Loring. Since tobacco and tomato plants are fairly closely related, it seemed of interest to determine whether protein isolated from a distantly related plant would possess the same sedimentation constant. An ultracentrifugal analysis of the protein obtained from mosaic-diseased phlox plants demonstrated that its sedimentation constant was the same as that of protein from tobacco plants diseased with the same virus strain.

It is important to know how much molecular destruction is brought about by the treatment of virus protein with chemical reagents capable of destroying its biological activity. Sedimentation constants on solutions of virus inactivated by hydrogen peroxide, formaldehyde, and nitrous acid are listed in Table I. In no case was there complete molecular disruption which would show itself through the presence of unsedimentable material, nor were the molecules split into a few large fragments. There were, however, differences in boundary diffuseness and the molecular homogeneity this indicates. The peroxide-treated molecules were as homogeneous as before, while those treated with  $\text{HNO}_2$  were so heterogeneous that only a rough estimate of a mean sedimentation constant was possible.

#### SUMMARY

A series of ultracentrifugal analyses by both the absorption and the refractive index methods has been made of solutions of the virus proteins derived from plants belonging to different plant families infected with different strains of tobacco mosaic virus.

The sedimentation constants of these proteins are the largest thus far found and correspond to molecules of a weight of several millions; otherwise, the results obtained are strictly analogous to those given by other large molecules.

Constants have been measured for these heavy molecules (1) as they occur in the untreated juice of infected plants, (2) in solutions of the crystalline mass obtained by centrifuging this juice at very high speeds, and (3) in solutions of the crystalline proteins isolated and purified by chemical methods. The fact that these constants are the same indicates that molecules of the same size are present in each preparation.

Heavy molecules were not found in the juice of healthy plants; furthermore, molecules with weights as great as 30,000 could not be detected in proteins precipitated from the juice from healthy plants with  $(\text{NH}_4)_2\text{SO}_4$ .

The molecular weight of the virus proteins does not change perceptibly in the pH range between approximately 2 and 9.3. On the acid side of pH 7 the true solubility falls very rapidly with decreasing pH, most of the protein being present as colloidal particles which often yielded very diffuse but observable boundaries with sedimentation constants of the order of 500. In solutions diluted to a concentration of 0.5 mg. of protein per cc. and allowed to stand, unsedimentable material appeared which indicates that under these conditions the large molecules may break up into smaller units. No such dissociation in stronger solutions has been observed even after several months standing.

Differences in rate of sedimentation exist between the virus proteins of different strains and between the proteins obtained at different times from plants inoculated with the virus of the ordinary strain.

Solutions of the virus proteins recovered from tobacco and tomato plants inoculated with the same virus strain and harvested at the same time and in the same way gave identical sedimentation constants. Virus proteins isolated from tobacco and phlox plants, representatives of two widely separated plant families, diseased with the same strain of mosaic virus were found to give the same sedimentation constant.

Treatment of the virus protein with  $\text{H}_2\text{O}_2$ ,  $\text{HCHO}$ , or  $\text{HNO}_3$  did

not disrupt the molecules, but in the case of the two latter reagents resulted in a distinct molecular heterogeneity.

Some of the virus proteins were molecularly homogeneous; others showed the broad and diffuse sedimenting boundaries that are indicative of a considerable molecular heterogeneity. Still others, giving double boundaries, contained two well defined molecular species.

These observations indicate that the virus proteins of the various strains of the tobacco mosaic disease consist of a group of related but definitely differing molecular species. Any simple relation that may exist between molecular shape and size and biologically specific characteristics can be told only after the possible virus strains have been more completely studied and standardized. It should be noted, however, that whenever wide differences exist in the sedimentation constants of two virus proteins, careful examination has revealed differences in the symptoms they call forth in inoculated plants.

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## THE EXCHANGE OF SALT AND WATER BETWEEN MUSCLE AND BLOOD

### I. THE EFFECT OF AN INCREASE IN TOTAL BODY WATER PRO- DUCED BY THE INTRAVENOUS INJECTION OF ISOTONIC SALT SOLUTIONS\*

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The successful application of the laws governing the behavior of electrolytes to the problem of the distribution of salts and water between the cells and plasma of blood, by Van Slyke and Henderson and their collaborators, stimulated the present authors some years ago to undertake a comparable study on the system consisting of skeletal muscle and blood serum.

In the past there have been numerous studies in which muscle has been analyzed for its water and inorganic constituents (1-7), but until recently the necessity for carrying out such analyses on muscle quickly removed from the living organism has not been generally appreciated. The techniques and the chemical laws which are applicable to blood are inapplicable to muscle because the conditions of muscle cell permeability, which are dependent upon metabolic processes, are quite different from those of blood cells. An excellent discussion of the electrolyte and water exchanges between tissue cells and interstitial fluids is to be found in Peters' recent book (8).

The system with which the present paper deals may be considered to consist of the following component parts.

|                       |   |                       |   |              |
|-----------------------|---|-----------------------|---|--------------|
| Muscle cells          | : | interstitial fluid    | : | blood plasma |
| (Intracellular phase) |   | (extracellular phase) |   | (serum)      |

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\* A report of the work discussed in this paper was presented at the meeting of the American Society of Biological Chemists at Detroit in March, 1935 (*Proc. Am. Soc. Biol. Chem.*, 8, xli (1935); *J. Biol. Chem.*, 109 (1935)).

"Muscle" as used in this paper denotes the intracellular phase plus the extracellular phase.

The specific purposes of the present study were (1) to determine the distribution of salts and water between serum and muscle under normal conditions; (2) to utilize these data for the quantitative estimation of the relative amounts of the extracellular and intracellular phases; (3) to determine the effect of an increase in total body water, produced by injection of isotonic salt solutions, on the relative and absolute volumes of the two muscle phases. These experiments were carried out (a) without alteration in the acid-base balance, (b) in alkalosis, and (c) in acidosis.

#### *General Properties of the System, Muscle-Serum*

Before presenting the detailed experiments, the assumptions concerning muscle which are pertinent to the present study will be enumerated.

1. Water moves freely across the barrier between the blood plasma and the extracellular phase, and across the barrier between the extracellular and intracellular phases. This results in osmotic equilibrium between all phases of the system.

2. Inorganic ions and small molecules move freely across the barrier between plasma and extracellular phase, but large molecules, notably the proteins of the plasma, do not. This results in an unequal distribution of inorganic ions which is determined by the Gibbs-Donnan distribution law.

3. Inorganic ions, as well as large molecules, under normal conditions, do not move across the barrier between the extracellular and intracellular phases of muscle. This condition of functional impermeability exists only as long as the normal metabolic state of the muscle is maintained. When metabolic processes cease, the barrier becomes permeable both to inorganic ions and to proteins.

4. Under normal metabolic conditions the intracellular phase of muscle contains no chloride. This cannot be determined by direct experiment; but the work to be presented in this paper, together with the information available from other sources, leads one to the conviction that this may be regarded as a reasonable assumption.

#### *Symbols and Equations*

By utilizing the foregoing assumptions and applying data obtained from determinations of the water and inorganic con-

stituents of serum and muscle, it is possible to estimate the relative proportions of the extra- and intracellular phases. The definitions of the symbols, and the equations used in the calculations, follow.

|              |   |  |
|--------------|---|--|
| $(Cl)_s$     | = | m.-eq. chloride per kilo serum         |
| $[Cl]_s$     | = | " " " " " water                        |
| $(Cl)_M$     | = | " " " " " muscle                       |
| $[Cl]_F$     | = | " " " " " extracellular phase          |
| $[Cl]_F$     | = | " " " " " water of extracellular phase |
| $[Cl]_C$     | = | " " " " " intracellular phase          |
| $(H_2O)_s$   | = | gm. water per kilo serum               |
| $(H_2O)_M$   | = | " " " " " muscle                       |
| $(H_2O)_F$   | = | " extracellular water per kilo muscle  |
| $(H_2O)_C$   | = | " intracellular " " " "                |
| $\{H_2O\}_F$ | = | " water per kilo extracellular phase   |
| $\{H_2O\}_C$ | = | " " " " " intracellular "              |
| $(F)$        | = | " extracellular phase per kilo muscle  |
| $(C)$        | = | " intracellular " " " "                |
| $M$          | = | $(F) + (C)$                            |

When constituents other than chloride or water have been referred to, comparable symbols have been used.

The equations used in the calculation of the extracellular phase  $(F)$  are

$$(F) = \frac{(Cl)_M}{\{Cl\}_F} \times 1000 \quad (1)$$

where 
$$\{Cl\}_F = \frac{0.99}{0.95} \times [Cl]_s$$

The figure 0.99 is taken as the water concentration in the extracellular phase in gm. of water per gm. of fluid. The figure 0.95 is the value of the ratio  $[Cl]_s/[Cl]_F$ .

To calculate the concentration of water in the intracellular phase

$$\{H_2O\}_C = \frac{(H_2O)_C}{(C)} = \frac{(H_2O)_M - (H_2O)_F}{(C)} \quad (2)$$

where 
$$(H_2O)_F = 0.99 \times (F)$$

$$(C) = 1000 - (F)$$

To calculate the change produced in the two phases of 1 kilo of original muscle by the injection of isotonic solutions, a further assumption was made; namely, that no change had been produced in the amount of solids in the intracellular phase.

$(S)_{c_1}$  = the solids present in the intracellular phase of 1 kilo of muscle, originally (denoted by the subscript 1)

$(S)_{c_2}$  = the solids present in the intracellular phase of 1 kilo of muscle, after injection of the solution (denoted by the subscript 2)

Then  $(S)_{c_1} + (H_2O)_{c_1} + (F)_1 = 1000$  before injection

and  $(S)_{c_2} + (H_2O)_{c_2} + (F)_2 = 1000$  after injection

If the solids of the intracellular phase have remained unchanged in amount, the final weight in gm. of the original kilo of muscle,  $M_f = ((S)_{c_1}/(S)_{c_2}) \times 1000$ ; the final weight of the intracellular phase,  $(C)_f = ((S)_{c_1}/(S)_{c_2}) \times (C)_2$ ; and the final weight of the extracellular phase,  $(F)_f = ((S)_{c_1}/(S)_{c_2}) \times (F)_2$ . The absolute change in the original kilo of muscle is then  $\Delta M = 1000 - M_f$ ; the change in the intracellular phase  $\Delta(C) = (C)_1 - (C)_f$ ; and the change in the extracellular phase  $\Delta(F) = (F)_1 - (F)_f$ . The significance of these equations will become clear after the presentation of the experimental data.

## EXPERIMENTAL

### *Physiologic Procedure*

The dogs used in these experiments were maintained under observation in metabolism cages for approximately 2 weeks and were in excellent physical condition at the time of experimentation.

Before an experiment was carried out, the dog was weighed and anesthetized by the intraperitoneal injection of 270 mg. of sodium barbital per kilo of body weight. (In a few experiments the animals were anesthetized with urethane or paraldehyde. However, these anesthetics were not found to be as satisfactory as barbital for the purposes of the present work.)

After about 1 hour the dog was placed on the board and the bladder catheterized. (Urine was collected throughout the experimental period.) A cannula was then introduced into the femoral artery. 50 cc. of blood were taken under oil, and one of the rectus abdominis muscles was removed, for the control analyses. All incisions were closed with hemostats, and the body was kept covered and warm. The salt solutions, warmed to 38°, were then injected by gravity through the femoral vein at the rate of 60 to 80 cc. per minute. The injections required from 30 to 40 minutes, after which 60 minutes were allowed to elapse for the

establishment of equilibrium between the blood and the muscle. The rectus abdominis muscle on the opposite side was then removed, and a second 50 cc. blood sample withdrawn, for the final analyses.

This study includes two groups of experiments, (1) uninjected animals and (2) animals receiving intravenous injections of 170 cc. per kilo of body weight of the following isotonic solutions: 129 mm of NaCl + 25 mm of NaHCO<sub>3</sub>, 114 mm of NaCl + 40 mm of NaHCO<sub>3</sub>, 154 mm of NaCl alone, 154 mm of NaCl + 10 mm of HCl.

The uninjected animals were subjected to the same procedures as the injected animals. After one of the rectus abdominis muscles was removed and the arterial blood taken, the dog was kept on the board covered and warm for 1 hour. The other muscle and the second blood sample were then taken.

The following determinations were made on the *serum*: pH, total CO<sub>2</sub>, water, chloride, protein, sodium, and total base; on the *muscle*: water, chloride, hemoglobin, sodium, total neutral fat, and total base. The hemoglobin of the whole blood was determined. In many experiments the serum and muscle were also analyzed for potassium.

The blood samples were centrifuged under oil and the serum removed for analysis. After the muscle had been wiped with sterile absorbent gauze to remove adherent blood, it was immediately put into a glass-stoppered weighing bottle. The muscle was shortly afterward placed on a tile and trimmed quickly to remove as much free fat and connective tissue as possible. It was wiped again, returned to the weighing bottle, and then finely minced with scissors. Weighed aliquot samples were used for all analyses. Each muscle was treated in exactly the same manner in every detail.

### *Chemical Methods*

All serum analyses were made in duplicate; all muscle analyses, in quadruplicate.

The *pH* of the serum was determined colorimetrically (9). *Carbon dioxide* was determined with the Van Slyke and Neill manometric gas apparatus (10).

*Chlorides* were determined on serum and muscle by the wet ashing method of Van Slyke (11) with the Wilson and Ball modifi-

cation (12). From 1.5 to 2.0 gm. of the finely minced and thoroughly mixed wet muscle were weighed, and then washed into large digestion tubes with 5 cc. of water. A slight excess of aqueous silver nitrate solution was added (1 cc. of 0.075 N) and the mixture was allowed to stand overnight. 4 cc. of concentrated nitric acid were then added and the material was digested until the solutions were clear. This usually required 2 to 3 hours, although muscle with a high percentage required a longer period for digestion. The titrations were carried out as on the serum. The results with this method were compared with those obtained by that of Sunderman and Williams (13), and showed good agreement.

*Sodium* determinations were made by the Butler-Tuthill method (14). The phosphate of the serum was removed by ignition with ferric sulfate according to Peters' procedure, and the sodium was determined on an aliquot from which the insoluble iron salt had been removed by centrifugation. The phosphate of the ignited muscle was removed by calcium hydroxide.

*Potassium* determinations were made by the Shohl-Bennett method (15). For the analyses of the potassium in muscle, approximately 2 gm. of muscle were dried overnight at 100°, and then ignited in the muffle furnace at 550°. The residue was then dissolved in 1 cc. of 1 N hydrochloric acid, and made up to 25 cc. volume, from which 2 cc. aliquots were evaporated in 10 cc. beakers. From this point the method was the same as that for serum.

*Total base* determinations in the serum were made by the method of Stadie and Ross (16), except that the phosphate was removed as in the sodium determinations. The total base of the muscle was determined as follows: 1 to 2 gm. of finely minced and mixed wet muscle were weighed into digestion tubes. 3 cc. of concentrated sulfuric acid and 1 cc. of concentrated nitric acid were added, and the contents digested to the charring stage. Concentrated nitric acid was then added until all organic matter was destroyed. The contents of the tubes were then washed into silica beakers and the water evaporated in an oven at 100°. The excess sulfuric acid was removed by heating the beakers on a sand bath until about 0.5 cc. of the sulfuric acid remained. The contents of the beakers were then transferred to 25 cc. volumetric flasks, neutralized with

freshly distilled dilute ammonia (approximately 1 N), and the phosphate removed by precipitation with ferric ammonium sulfate. The excess ferric ion was precipitated with ammonium hydroxide, and the mixture was then diluted to the 25 cc. mark and filtered. 20 cc. of the filtrate and 0.5 cc. of concentrated sulfuric acid were returned to the silica beakers, the water evaporated, and the contents ignited in a muffle furnace at 550°, until all ammonium salts and free sulfuric acid had been removed. From this point the method as applied to serum was followed.

The *hemoglobin* determinations were made by using the control blood and muscle as standards. The blood and muscle taken after the intravenous injection of the salts were compared with the standards by the method of Cohen and Smith (17). To determine the hemoglobin in muscle, 2 gm. of the finely minced muscle were ground in a mortar with fine, washed dry sand and 5 cc. of water. The mixture was centrifuged and the supernatant fluid used as in the blood determinations. The hemoglobin is reported in terms of the relative rather than the absolute amount present.

The *water* contents of serum and muscle in gm. of water per gm. of serum or muscle were obtained by drying known weights of serum and muscle to constant weight at 110° in weighing bottles.

The *neutral fat* was extracted from the muscle by the following procedure. 5 cc. of dry ethyl ether were added to each of the weighing bottles containing the muscle residue remaining after the evaporation of water. The mixture was stirred and allowed to stand for 10 minutes, after which the ether layer was drawn off by a fine tipped pipette. Extraction with ether was repeated, after which the ether was entirely removed by evaporation. The muscle residue was then extracted with petroleum ether (boiling point 40–60°) until the residue reached constant weight. This resulted after six to eight extractions. The difference between the weights of the residue before and after the extraction gave the weight of fat in the sample of muscle taken.

*Serum proteins* were determined by the micro-Kjeldahl method. They were estimated by multiplying by 6.25 the total nitrogen, corrected for non-protein nitrogen.

Special reasons for carrying out some of the analyses are as follows: (1) The pH and CO<sub>2</sub> of serum were determined to evaluate the acid-base balance of the blood before and after the injec-



tion of the various solutions. (2) The fat content of the muscle was determined in order that all of the analytical results on muscle might be expressed in terms of concentration units per kilo of fat-free muscle. Before this was done it was impossible to correlate the muscle values from different dogs, or even from different muscles of the same dog. It will be seen from the tables, however, that when the results are expressed in terms of fat-free muscle there is great uniformity both with respect to water content and to the concentration of the inorganic constituents. (3) The hemoglobin of the blood and muscle was determined in many experiments in order to estimate the relative amounts of blood in the muscle at the beginning and end of the experiments. It was found that in most of the experiments the decrease of the hemoglobin content in the muscle approximately paralleled that in the blood, indicating thereby that the changes observed in the water content of the muscle were not to be attributed to an increase or decrease of its content of blood.

### *Results*

#### *Analytical Data on Serum and Muscle of Normal Dogs*

*Values for Constituents of Muscle and Serum*—A summary of the data obtained from the analysis of the serum and muscle of twenty normal dogs is given in Table I. The points of interest in the data are: (a) Not only was the mean value of the water content of the original muscle, without extraction of fat, much lower than the value on a fat-free basis (680 compared with 765 gm. per kilo), but the standard deviation was much larger (77 compared with 6.4σ). The variation of the water content of the muscle expressed on a fat-free basis was hardly greater than that found in the serum. (b) The variations in the concentrations of total base, sodium, and chloride of muscle were not markedly different from the variations of the same constituents of serum. Had the concentrations not been expressed in terms of fat-free muscle, the variation from animal to animal would have been much greater.

From these data, it may be concluded that in the study of water and electrolyte relations in tissues it is essential to express the results in terms of fat-free tissue.

*Extracellular and Intracellular Phases of Normal Muscle*—From the data just presented, and the assumption that all of the muscle

chloride is in the extracellular phase, it was possible to calculate from Equations 1 and 2 the relative proportions of the extra- and intracellular phases of muscle. Such a calculation led to the following results: extracellular phase ( $F$ ) = 173 gm.,  $\sigma = 22$ ; intracellular phase ( $C$ ) = 827 gm.,  $\sigma = 22$ . These data were also used to calculate the per cent of water in the intracellular phase. This led to the value for  $\{H_2O\}_C$  of 717 gm. per kilo of muscle cells;  $\sigma$ , 5. In view of the general opinion that the percentage of muscle water is subject to wide variation, the constancy of the

TABLE I  
*Normal Values for Dog Serum and Muscle*

The figures are averages from twenty dogs. The rectus abdominis muscle was used. The results on muscle are expressed as units per kilo of fat-free muscle.  $\sigma$ , standard deviation.

| Constituent                        | Serum |          | Muscle |          |
|------------------------------------|-------|----------|--------|----------|
|                                    | Mean  | $\sigma$ | Mean   | $\sigma$ |
| Total base, m.-eq. per kilo.....   | 152   | 3.9      | 143    | 6.2      |
| Chloride " " " .....               | 109   | 1.7      | 21.5   | 2.8      |
| Sodium " " " .....                 | 142   | 4.1      | 32.4   | 4.8      |
| Potassium " " " .....              | 3.72  | 0.4      | 82.1   | 10.0     |
| Total protein, gm. " " .....       | 57.0  | 4.7      |        |          |
| Non-protein N, " " " .....         | 0.32  | 0.07     |        |          |
| CO <sub>2</sub> , mm per kilo..... | 24.4  | 2.4      |        |          |
| pH.....                            | 7.40  | 0.06     |        |          |
| Water, gm. per kilo*               | 922   | 6.0      | 765    | 6.4      |
| " " " " †                          |       |          | 680    | 77.0     |

\* Corrected for fat.

† Uncorrected for fat.

water of the intracellular phase, when allowance is made for the fat content of the muscle and for the magnitude of the extracellular phase, is worthy of special emphasis.

*Graphic Representation of Electrolyte and Water Equilibria between Serum and Muscle*—From the data of Table I, and the estimates of the extra- and intracellular phases of muscle, the concentrations and total amounts of total base, sodium, potassium, chloride, water, and solids in the two phases have been calculated. These data are shown graphically in Fig. 1.

Fig. 1 shows the quantitative relations existing between the

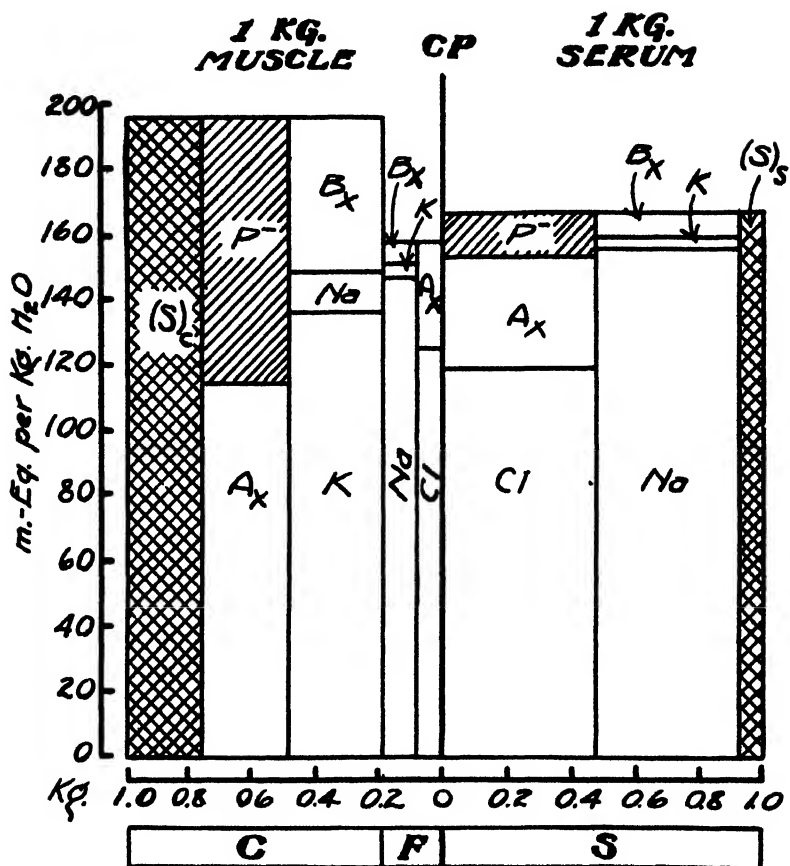


FIG. 1. Graphic representation of electrolyte and water equilibria between serum and muscle. Concentrations are expressed in milli-equivalents per kilo of water along the ordinate; absolute amounts of the components of the system are expressed in kilos along the abscissa. The line CP indicates the capillary barrier between the blood serum and the extra-cellular phase. The unshaded areas on the ordinate show the concentrations of the electrolytes. The simple cross-hatched areas show the approximate concentrations of non-diffusible protein anions,  $P^-$ . Unspecified anions and cations are designated by  $A_x$  and  $B_x$ , respectively. Along the abscissa, the double cross-hatched portions designate the kilos of solids in the intracellular phase and serum respectively; the unshaded areas, the kilos of water in the respective phases.

intra- and extracellular phases of 1000 gm. of normal dog muscle and 1000 gm. of blood serum. Although the amount of ionized serum protein is approximately correct, the amount estimated for muscle is as yet uncertain. Its accurate evaluation awaits further information on the titration curves of muscle proteins and the degree of ionization of their salts. The following relations between serum and muscle have been illustrated in Fig. 1.

*Between Serum and Extracellular Phase*—(1) Electrolyte neutrality, shown by the equality of the heights of the cation and anion areas. Unspecified anions and cations are designated by  $A_x$  and  $B_x$  respectively. (2) Osmotic equilibrium, shown by the equality of the sum of the heights of the unshaded portions of the anion and cation areas of the serum and the extracellular phase respectively. (3) Gibbs-Donnan equilibrium, shown by the designated heights of the cation and anion areas separately. These have been plotted in such a way that

$$\frac{[Na]_F}{[Na]_s} = \frac{[Cl]_s}{[Cl]_F} = \frac{[B]_F}{[B]_s} = \frac{[A]_s}{[A]_F} = 0.95$$

(4) The indicated relation between the total amount of extracellular phase and total amount of serum is arbitrary. The designated relation between the solids and water of serum rests on experimental evidence.

*Between Extracellular and Intracellular Phases*—(1) Electrolyte neutrality, shown by the equality of the heights of the cation and anion areas. (2) Osmotic equilibrium, shown by the equality of the sum of the heights of the unshaded portions of the anion and cation areas of the extra- and intracellular phases. (3) The *absence* of a Gibbs-Donnan equilibrium in the sense in which it exists between extracellular fluid and serum or between the serum and cells of blood. (4) The width of the area of the extracellular phase on the  $X$  axis shows the maximum value which it could have and be consistent with our data (173 gm. per kilo of muscle). A larger extracellular phase would require the presence of a greater amount of chloride in muscle than is actually found by analysis. (5) The widths of the cross-hatched and unshaded areas of muscle along the  $X$  axis are not arbitrary but are determined by the analytical data.

*Conclusion*—Normal fat-free skeletal muscle of dogs consists

of an extracellular phase amounting to a maximum of 17 per cent and an intracellular phase amounting to 83 per cent of the muscle. This is not an unreasonable physical proportion of the two phases. Such a conclusion involves the assumption that there is no chloride and very little sodium in the intracellular phase, and that under normal metabolic conditions there is no exchange of sodium, potassium, or chloride ions between the intra- and extracellular phases. It is assumed that water, on the other hand, passes freely from one phase to the other to maintain osmotic equilibrium. Under normal conditions the per cent of water in the intracellular phase is remarkably constant.

TABLE II

*Analyses of Serum and Muscle before and after Equilibration at 38°*

Time equilibrated, 3½ hours. Final pH, 7.32; CO<sub>2</sub>, 29.8 mm per liter of serum. Muscle values corrected for fat.

|   |        | H <sub>2</sub> O  | Cl                                    | Na                                    | K                                     | $\frac{[Cl]_M}{[Cl]_S}$ | $\frac{[Na]_S}{[Na]_M}$ | $\frac{[K]_S}{[K]_M}$ |
|---|--------|-------------------|---------------------------------------|---------------------------------------|---------------------------------------|-------------------------|-------------------------|-----------------------|
|   |        | gm.<br>per<br>gm. | m.-eq.<br>per kg.<br>H <sub>2</sub> O | m.-eq.<br>per kg.<br>H <sub>2</sub> O | m.-eq.<br>per kg.<br>H <sub>2</sub> O |                         |                         |                       |
| Immediately after<br>removal, before<br>equilibration             | Serum  |                   | 114.3                                 | 147.0                                 | 3.9                                   | 0.19                    | 4.3                     | 0.031                 |
|   | Muscle | 0.764             | 21.0                                  | 34.4                                  | 124.0                                 |                         |                         |                       |
| After addition of<br>NaHCO <sub>3</sub> , before<br>equilibration | Serum  | 0.944             | 81.8                                  | 151.0                                 | 3.6                                   | 0.98                    | 0.88                    | 0.5                   |
|   | Muscle | 0.836             | 68.6                                  | 125.4                                 | 30.0                                  |                         |                         |                       |

Similar conclusions regarding the salt and water distribution between the extra- and intracellular phases of muscle have been reached by Peters (18) and by Harrison, Darrow, and Yannet (19). Since their calculations were not based on fat-free tissue, as in the present paper, the magnitudes of the extra- and intracellular phases are somewhat different from those reported here.

#### *Effect of Equilibration in Vitro on Equilibria between Serum and Muscle*

As is well known, any conditions which lead to injury of muscle cells give rise to greatly altered permeability. An experiment

demonstrating this in an exaggerated form is shown in Table II. Muscle and serum were removed from a dog as previously described. They were then analyzed for their water, chloride, sodium, potassium, and protein concentrations before and after equilibration for 3.5 hours at 38°. Before the equilibration was begun, 40 cc. of 0.154 M sodium bicarbonate were added to 100 cc. of serum, an amount which had been determined by previous experiments to be necessary to allow for neutralization of the acid formed during the equilibration. In the experiment cited, the pH of the serum was initially 7.40; at the end of the equilibration it was 7.32. It is seen from the last three columns of Table II that

TABLE III

*Analyses of Serum and Muscle of Uninjected Control Animals*

Muscle concentrations are expressed in units per kilo of original muscle, not corrected for fat.

|  |        |         | pH   | CO <sub>2</sub> | H <sub>2</sub> O | Cl             | Na             | Total base     | (F) |
|--|--------|---------|------|-----------------|------------------|----------------|----------------|----------------|-----|
|  |        |         |      | mm per kg.      | gm. per gm.      | m.-eq. per kg. | m.-eq. per kg. | m.-eq. per kg. |     |
| Dog 20;<br>weight 9<br>kilos; time<br>1 hr.  | Serum  | Initial | 7.42 | 23.80           | 0.9167           | 113            | 150            | 169            |     |
|  |        | Final   | 7.51 | 22.91           | 0.9171           | 114            | 148            | 171            |     |
|  | Muscle | Initial |      |                 | 0.7354           | 22.94          | 36.18          |                | 175 |
|  |        | Final   |      |                 | 0.7305           | 23.45          | 35.13          |                | 174 |
| Dog 21;<br>weight 15<br>kilos; time<br>1 hr. | Serum  | Initial | 7.48 | 28.20           | 0.9224           | 110            | 146            | 165            |     |
|  |        | Final   | 7.47 | 26.60           | 0.9247           | 114            | 147            | 166            |     |
|  | Muscle | Initial |      |                 | 0.7438           | 26.20          | 34.75          |                | 206 |
|  |        | Final   |      |                 | 0.7379           | 26.81          | 33.95          |                | 206 |

the distribution ratios of chloride, sodium, and potassium in serum and muscle bore no relation to one another before equilibration. After equilibration for 3.5 hours and the resulting death of the muscle, water, chloride, and sodium had entered the muscle from the serum, and potassium had entered the serum from the muscle, until a state approaching that expected from the Gibbs-Donnan distribution law existed.

It may be concluded from the experiment cited that muscle under normal metabolic conditions is impermeable to the inorganic ions chloride and sodium. Numerous other experiments on the distribution of sodium and chloride after equilibration of muscle

and serum at different pH values were carried out. Changes in the distribution ratios were found, which, though small, were in the direction predicted by the Gibbs-Donnan law. Since these experiments have no apparent physiological significance, their details have been omitted from this paper.

TABLE IV

*Changes in Serum and Muscle after Injection of Normal Isotonic Solution*

Solution, 25 mm of  $\text{NaHCO}_3$  + 129 mm of  $\text{NaCl}$ . Muscle values corrected for fat.

|   |         | pH   | $\text{CO}_2$ | Hb  | Plasma protein | $\text{H}_2\text{O}$ | K     | Cl    | Na    | Total base | (F) |
|---|---------|------|---------------|-----|----------------|----------------------|-------|-------|-------|------------|-----|
| Dog 62; weight 17 kilos; 2800 cc. injected; 255 cc. urine |         |      |               |     |                |                      |       |       |       |            |     |
| Serum   | Initial | 7.43 | 28.23         | 100 | 53.3           | 0.9260               | 4.10  | 111.1 | 142.0 | 153        |     |
|   | Final   | 7.42 | 26.13         | 67  | 33.2           | 0.9504               | 3.43  | 119.0 | 146.0 | 155        |     |
| Muscle  | Initial |      |               |     |                | 0.7622               | 91.50 | 19.82 | 33.18 | 153        | 158 |
|   | Final   |      |               |     |                | 0.7704               | 83.70 | 24.21 | 36.54 | 155        | 185 |
| Dog 63; weight 29 kilos; 3000 cc. injected; 275 cc. urine |         |      |               |     |                |                      |       |       |       |            |     |
| Serum   | Initial | 7.42 | 26.76         | 100 | 57.8           | 0.9217               | 4.29  | 110.5 | 147.0 | 157        |     |
|   | Final   | 7.42 | 26.16         | 69  | 31.5           | 0.9499               | 3.50  | 118.4 | 147.1 | 155        |     |
| Muscle  | Initial |      |               |     |                | 0.7675               | 73.40 | 20.27 | 32.20 | 134        | 163 |
|   | Final   |      |               |     |                | 0.7789               | 74.20 | 26.30 | 39.07 | 146        | 202 |
| Dog 64; weight 20 kilos; 3000 cc. injected; 380 cc. urine |         |      |               |     |                |                      |       |       |       |            |     |
| Serum   | Initial | 7.38 | 25.67         | 100 | 57.3           | 0.9197               | 4.15  | 107.6 | 139.4 | 151        |     |
|   | Final   | 7.37 | 26.79         | 67  | 35.5           | 0.9461               | 2.93  | 114.2 | 140.2 | 151        |     |
| Muscle  | Initial |      |               |     |                | 0.7708               | 88.64 | 22.32 | 39.13 | 156        | 183 |
|   | Final   |      |               |     |                | 0.7828               | 88.00 | 24.80 | 40.13 | 153        | 197 |

### Control Animals

Table III shows the results of two typical experiments on normal animals which served as uninjected controls. The animals were anesthetized, blood and muscle were removed for analysis, and an hour was allowed to elapse before the second samples of blood and muscle were removed. No differences in the serum or muscle of quantitative significance were observed, nor was any difference

TABLE V

*Changes in Serum and Muscle after Injection of Alkaline Isotonic Solution*Solution, 114 mm of NaCl + 40 mm of NaHCO<sub>3</sub>. Muscle values corrected for fat.

|   |         | pH   | CO <sub>2</sub> | Hb  | Plasma protein | H <sub>2</sub> O | Cl    | Na    | Total base | (F) |
|---|---------|------|-----------------|-----|----------------|------------------|-------|-------|------------|-----|
| Dog 51; weight 13 kilos; 2300 cc. injected; 474 cc. urine |         |      |                 |     |                |                  |       |       |            |     |
| Serum   | Initial | 7.42 | 23.20           | 100 | 51.0           | 0.9326           | 108.0 | 140.0 | 148        |     |
|   | Final   | 7.61 | 27.60           | 50  | 29.6           | 0.9568           | 113.6 | 146.5 | 156        |     |
| Muscle  | Initial |      |                 |     |                | 0.7555           | 18.97 | 24.76 | 141        | 157 |
|   | Final   |      |                 |     |                | 0.7845           | 27.66 | 38.08 | 143        | 223 |
| Dog 53; weight 11 kilos; 1900 cc. injected; 83 cc. urine  |         |      |                 |     |                |                  |       |       |            |     |
| Serum   | Initial | 7.52 | 28.22           | 100 | 51.0           | 0.9294           | 107.0 | 140.9 | 151        |     |
|   | Final   | 7.62 | 31.44           | 75  | 24.8           | 0.9612           | 112.8 | 148.8 | 155        |     |
| Muscle  | Initial |      |                 | 100 |                | 0.7689           | 21.58 | 24.35 | 139        | 180 |
|   | Final   |      |                 | 97  |                | 0.7942           | 27.77 | 37.04 | 151        | 227 |

TABLE VI

*Changes in Serum and Muscle after Injection of Isotonic Sodium Chloride*

Solution, 154 mm of NaCl. Muscle values corrected for fat.

|   |         | pH   | CO <sub>2</sub> | Hb  | Plasma protein | H <sub>2</sub> O | Cl    | Na    | Total base | (F) |
|---|---------|------|-----------------|-----|----------------|------------------|-------|-------|------------|-----|
| Dog 55; weight 11 kilos; 1900 cc. injected; 130 cc. urine |         |      |                 |     |                |                  |       |       |            |     |
| Serum   | Initial | 7.36 | 20.96           | 100 | 57.6           | 0.9190           | 110.4 | 145.7 | 156        |     |
|   | Final   | 7.27 | 16.08           | 60  | 27.0           | 0.9575           | 128.2 | 146.8 | 154        |     |
| Muscle  | Initial |      |                 | 100 |                | 0.7669           | 24.46 | 36.10 | 136        | 195 |
|   | Final   |      |                 | 80  |                | 0.7849           | 35.40 | 47.27 | 141        | 254 |
| Dog 56; weight 21 kilos; 3500 cc. injected; 950 cc. urine |         |      |                 |     |                |                  |       |       |            |     |
| Serum   | Initial | 7.40 | 24.30           | 100 | 61.7           | 0.9166           | 108.3 | 143.1 | 153        |     |
|   | Final   | 7.40 | 19.12           | 75  | 40.8           | 0.9416           | 123.3 | 146.1 | 153        |     |
| Muscle  | Initial |      |                 | 100 |                | 0.7627           | 22.60 | 33.13 | 137        | 184 |
|   | Final   |      |                 | 77  |                | 0.7806           | 31.41 | 39.54 | 142        | 236 |



found in the calculated amounts of the extracellular phase of the muscle.

### *Injection of Isotonic Salt Solutions*

The effect of the intravenous injection of salt solutions on the intra- and extracellular phases of muscle was then studied. Such experiments may be regarded as a study of acute edema of muscle tissue, and fall into three groups: (1) "normal" experiments, those in which the injected salt solutions contained 25 mm per liter of

TABLE VII

*Changes in Serum and Muscle after Injection of Acid Isotonic Solution*

Solution, 10 mm of HCl + 154 mm of NaCl. Muscle values corrected for fat.

|   |         | pH   | CO <sub>2</sub> | Hb       | Plasma protein | H <sub>2</sub> O | K              | Cl             | Na             | Total base     | (F) |
|---|---------|------|-----------------|----------|----------------|------------------|----------------|----------------|----------------|----------------|-----|
| Dog 57; weight 11 kilos; 2000 cc. injected; 278 cc. urine |         |      |                 |          |                |                  |                |                |                |                |     |
|   |         |      | mm per l.       | per cent | gm. per kg.    | gm. per gm.      | m.-eq. per kg. | m.-eq. per kg. | m.-eq. per kg. | m.-eq. per kg. |     |
| Serum   | Initial | 7.37 | 21.55           | 100      | 54.5           | 0.9220           | 3.72           | 109.3          | 136.0          | 151            |     |
|   | Final   | 7.26 | 14.17           | 70       | 31.6           | 0.9458           | 3.37           | 127.2          | 139.0          | 152            |     |
| Muscle  | Initial |      |                 | 100      |                | 0.7756           |                | 25.08          | 32.96          | 135            | 203 |
|   | Final   |      |                 | 80       |                | 0.7904           |                | 30.93          | 37.02          | 140            | 221 |
| Dog 58; weight 28 kilos; 3500 cc. injected; 370 cc. urine |         |      |                 |          |                |                  |                |                |                |                |     |
| Serum   | Initial | 7.40 | 26.28           | 100      | 63.9           | 0.9160           | 3.73           | 106.6          | 143.1          | 152            |     |
|   | Final   | 7.22 | 15.31           | 61       | 30.6           | 0.9540           | 3.54           | 128.3          | 147.5          | 152            |     |
| Muscle  | Initial |      |                 |          |                | 0.7716           | 94.20          | 19.00          | 33.34          | 147            | 157 |
|   | Final   |      |                 |          |                | 0.7851           | 89.04          | 24.30          | 36.42          | 155            | 173 |

sodium bicarbonate, so that, although the amount of solution injected was large, there was no alteration in the acid-base balance (Table IV); (2) "alkalosis" experiments, those in which a moderate alkalosis was produced by increasing the sodium bicarbonate of the injected solution to 40 mm per liter (Table V); (3) "acidosis" experiments, those in which an acidosis was produced either by the injection of isotonic sodium chloride, containing no bicarbonate (Table VI), or of isotonic sodium chloride containing 10 mm of hydrochloric acid per liter (Table VII).

In all of the experiments there were found (1) a decrease in the hemoglobin content of the blood and the proteins of the serum and (2) an increase in the water, chloride, and sodium content of the muscle.

The correlation between the increases in sodium and chloride of the muscle with the changes in water content is shown in Fig. 2. Each experiment is represented by a line, and an arrow indicates

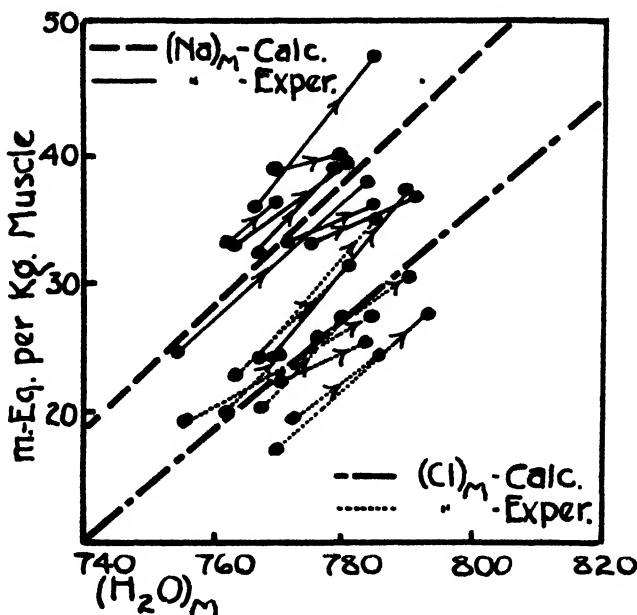


FIG. 2. Theoretical and experimental correlations between sodium and total water, and between chloride and total water, in muscle. Each experiment is represented by a line, the arrow indicating the direction of the change produced.

the direction of the change produced. The heavy broken lines denote the theoretical changes which would have been produced, assuming that the increase in the sodium and water, on the one hand, and in the chloride and water, on the other, was the result of simply increasing the bulk of the muscle by the progressive addition of an ultrafiltrate of blood plasma to a fixed amount of intracellular phase. The directions of the changes observed in the sodium, chloride, and water are consistent with this hypothesis.

*Calculations of Changes Produced in Muscle Phase*

From the data of Tables IV to VII, and the equations previously given, the relative changes of extra- and intracellular phases before and after the injection of the salt solutions have been calculated. In all cases there was an increase in the extracellular phase, the amount of the increase varying from 14 gm. per kilo of muscle in one of the normal experiments to 66 gm. per kilo in one of the alkalosis experiments. In three normal experiments the average increase of extracellular phase was 26 gm., or 16 per cent; in three alkalosis experiments, the average increase was 53 gm., or 33 per cent; in five acidosis experiments the average increase was 40 gm., or 23 per cent.

Since there is more physiologic significance in the absolute changes in extra- and intracellular phases than in their relative changes, these changes have been estimated in our experiments. The mathematical basis of the calculations has been presented in a preceding section. It may be recalled in this connection that an additional assumption is involved; namely, that as a result of the injection of the isotonic salt solutions there had been no alteration in the amount of solids present in that quantity of intracellular phase originally present in 1 kilo of muscle.

The results of these calculations are graphically presented in Fig. 3, which shows the absolute increase produced in the bulk of 1 kilo of muscle by the injection of the various solutions employed.

In the normal experiments the absolute changes amounted to an average of 48 gm. per kilo of original muscle, consisting of a 36 gm. increase in extracellular phase and a 12 gm. increase (a negligible amount) in intracellular phase. From these data one may conclude that an increase in the total body water produced by the rapid injection of an isotonic sodium chloride-sodium bicarbonate solution, which does not change the acid-base balance of the blood, produces a tissue edema which is entirely accounted for by an increase in extracellular phase; *i.e.*, without swelling or shrinking of intracellular phase.

In the alkalosis experiments the increase produced in 1 kilo of original muscle in Dogs 51 and 53 was large, averaging 126 gm., and consisting of an average increase of 41 gm. in intracellular phase and of 85 gm. in extracellular phase. These data tend to substantiate quantitatively a postulate familiar to clinicians;

namely, that alkalis accentuate the severity of an edema. The same number of moles of sodium and the same amount of water were injected into the animals of this group as into those of the normal group. The excretion of the injected salt, as estimated

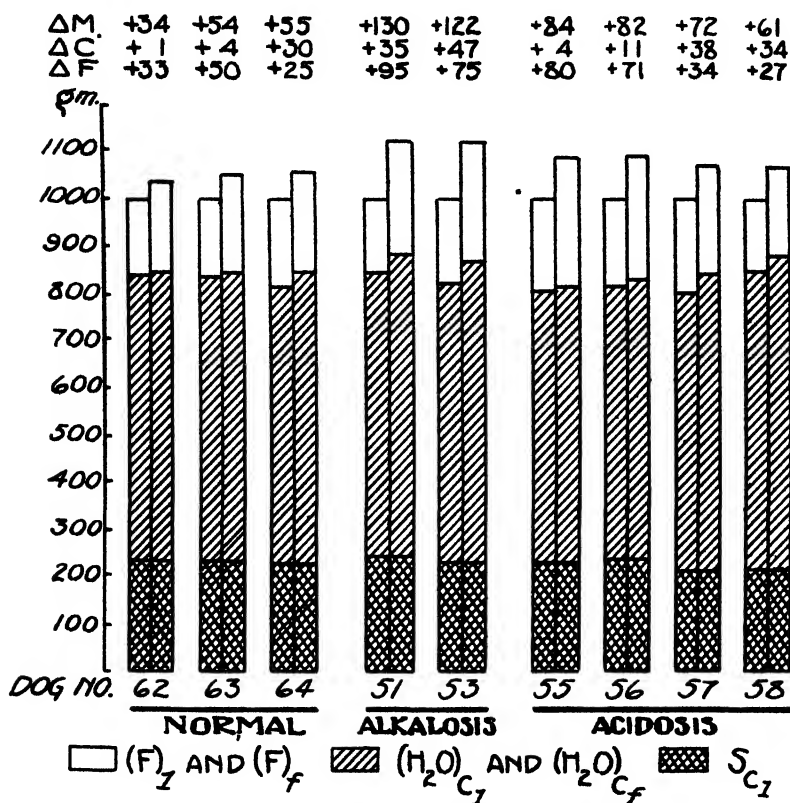


FIG. 3. Intracellular water and solids and extracellular phase before and after experimental procedures, showing absolute changes. The first column in each pair presents the original data, and the adjoining column presents the final data, for one experiment.

from chloride determinations, amounted to 13 and 14 per cent of the amount administered in the two groups respectively. Nevertheless, the increase in the bulk of the muscle in the alkalosis experiments was approximately 3-fold that found in the normal experiments. One-third of this increase is to be attributed to the

swelling of the cells and two-thirds to an increase in the amount of interstitial fluid.

The acidosis experiments were in two groups, one in which isotonic sodium chloride without added acid was injected (Dogs 55 and 56), and the other in which sodium chloride plus added hydrochloric acid was injected (Dogs 57 and 58). The increases observed in the muscles of the first group averaged 83 gm. and were due essentially to increases in extracellular phase. In the second group, the increases in the muscle were less than in the first, 67 compared with 83 gm., and showed an increase in intracellular phase amounting to 36 gm.

#### SUMMARY

1. A method is presented for the study of the shifts of water and salts between muscle and blood in the living dog.

2. Average normal values for sodium, chloride, water, potassium, and total base in dog serum and skeletal muscle have been determined.

3. The total water content of normal, fat-free skeletal muscle was found to be 76.5 per cent; the percentage of water in the intracellular phase of muscle was found to be 71.7.

4. From the analytical data obtained on normal dogs and the assumption that all of the muscle chloride is in the extracellular phase, the relative proportions of extra- and intracellular phases of normal, fat-free skeletal muscle have been calculated. The extracellular phase amounts to a maximum of 17 per cent and the intracellular phase amounts to 83 per cent of the muscle.

5. Experiments on dogs are described in which the extra- and intracellular phases of muscle were studied after an increase in the total body water produced by the rapid injection of large volumes of isotonic salt solutions (1) without altering the acid-base balance, (2) in alkalosis, and (3) in acidosis. From these data the following conclusions were reached: (a) The amount of extracellular phase in muscle increases, without swelling or shrinking of the intracellular phase, when a balanced isotonic salt solution is injected. (b) The amount of extracellular phase in muscle increases greatly and the intracellular phase swells appreciably when an alkaline isotonic salt solution is injected. (c) The amount of extracellular phase in muscle increases and the intracellular phase swells slightly when an acid isotonic salt solution is injected.

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# STUDIES ON THE CONSTITUTION OF INSULIN\*

## I. PROPERTIES OF REDUCED INSULIN PREPARATIONS

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Insulin<sup>1</sup> is a protein, related to the class of albumins. It is rich in sulfur (3.2 per cent), containing 8 to 9 per cent of cystine. No other sulfur-containing constituent has as yet been detected or isolated (2-4). The fully active hormone contains no free sulfhydryl groups. All of the insulin sulfur appears to be present in the form of dithio ( $\text{—S—S—}$ ) linkages; agents which reduce or oxidize these disulfide groups are reported to inactivate the hormone (1, 5).

The activity of an increasing number of biologically important substances, *e.g.* pitressin, pitocin, papain, cathepsin, urease, seems to depend largely on their state of oxidation-reduction or, more precisely, on the position of their  $\text{S—S} \rightleftharpoons 2\text{SH}$  equilibrium. Insulin belongs to this group.

The methods devised by Mirsky and Anson (6, 7) are employed in part in the present study; they permit a chemical determination of the state of oxidation-reduction of the sulfur in proteins and a correlation with the existence of the protein in the native or denatured state. The specific interaction of sulfhydryl compounds and proteins has previously been employed for a study of the inactivation of insulin by cysteine and glutathione (8, 9). The objectives of the present experiments were: to obtain specific information concerning the distribution of the sulfur in the hor-

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<sup>1</sup> For a review of the literature prior to 1934 see (1).



none molecule; to study its reduction behavior; to assess the contribution of the insulin sulfur to the physiological activity.

#### EXPERIMENTAL

##### *Insulin Preparations*

In most of the present experiments highly purified, amorphous insulin was used. This material, prepared by Eli Lilly and Company (Batch W-1282), had an activity of 20 to 22 units per mg. Determinations of the total sulfur content by the wet oxidation method yielded an average value of 3.2 per cent. The insulin used approaches, therefore, the crystalline preparations of Abel and coworkers (10) and of Scott (11), with respect to both physiological activity (24 units per mg.) and sulfur content (3.2 per cent). Originally, it was planned to make extensive use of crystalline insulin prepared by Scott's zinc method (12). This crystalline product, however, was found to be much less soluble than the amorphous material. It was, indeed, impossible to obtain solutions containing appreciable amounts of crystalline insulin with water or with buffer solutions of widely varying pH. Dr. D. A. Scott, in a private communication, was kind enough to corroborate this observation from his own experience. He suggested the use of strong acid or alkali to effect solution of the material. With this technique it is possible to prepare solutions of sufficient insulin concentration. However, since objections may possibly be raised against this procedure in studies of the native hormone, only a few experiments were conducted with crystalline insulin.<sup>2</sup> Most of the work was done with the purified insulin powder, which does not require preliminary treatment with the agents mentioned.

##### *Experiments with Thioglycolic Acid As Reducing Agent*

Preliminary experiments with various reducing agents confirmed the statement of Freudenberg and Wegmann (5) that thioglycolic acid appears to be the most effective reagent for the reduction of disulfide linkages in the insulin molecule. Mirsky and Anson (6, 7) obtained similar evidence in studies of other proteins.

<sup>2</sup> Scott's procedure for the preparation of crystalline insulin was chosen as the only method readily capable of yielding quantities required by this investigation. However, it must be kept in mind that, according to Scott, this product is probably the zinc salt of insulin.

Thioglycolic acid was therefore selected for a quantitative study of the reduction of the hormone. For the initial experiments, a purified sample of thioglycolic acid (b.p.  $97-99^{\circ}$  at 9 mm.) was used; this was kindly supplied by Dr. Maxwell P. Schubert. For

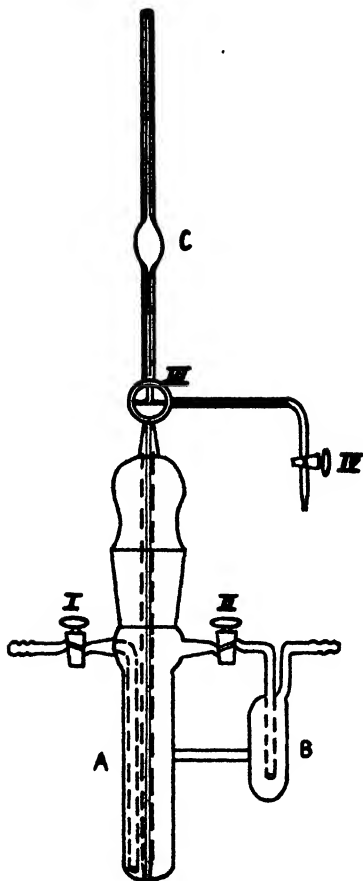


FIG. 1. Reduction apparatus. An explanation of the letters and numerals is given in the text.

the majority of the quantitative experiments, a thioglycolic acid preparation of Eastman was used after redistillation *in vacuo*.

*Technique of Reduction*—The Pyrex reduction apparatus employed is shown in Fig. 1. The capacity of the vessel (A) up to

the bulb is 30 cc. The tube leading to tap *I* is connected with a supply of oxygen-free nitrogen. The reduction vessel is immersed up to the taps *I* and *II* in a water thermostat at 30°. Usually, the main vessel was filled with 10 cc. of the buffered insulin solution and a drop of caprylic alcohol added to prevent frothing. The side vessel (*B*) contains water and serves as a gas trap. Before the reducer is added, nitrogen is passed through the insulin solution for at least 30 minutes. The reducer is added through a small funnel fixed on top of the burette (*C*). For the withdrawal of samples at various intervals, tap *II* is closed and the nitrogen pressure temporarily increased. By suitable manipulation of taps *III* and *IV* the mixture is first allowed to rise above the upper mark in the burette and then to fill completely, down to the tip, the tube leading to tap *IV*. The level of the solution is accurately adjusted to the upper mark and fluid is withdrawn by opening tap *IV* until the lower mark is reached. The burette is calibrated to deliver 1.0 cc. between the two marks. The remainder of the solution is returned to the reduction vessel. If the entire amount of solution in the reduction chamber is to be withdrawn after a certain time, direct communication between the vessel and tap *IV* is established by tap *III*, tap *II* is closed, and the solution is forced out by nitrogen pressure. The burette ends directly above the bottom of the chamber; the solution may therefore be removed almost quantitatively.

*Removal of Thioglycolic Acid*—If quantitative determinations of thiol groups formed in insulin are to be made, the excess of thioglycolic acid must be completely removed. Ether extraction (5) and prolonged washing with dilute trichloroacetic acid (6, 7) have been recommended for this purpose. Both procedures were found to be unsatisfactory if large volumes or high protein concentrations are involved. Continuous extraction of concentrated insulin solutions with ether leads to denaturation, even when the Soxhlet type of extractor is replaced by an arrangement permitting the cooling of the protein solution during extraction. The trichloroacetic acid procedure involves handling of large volumes of washing fluid and is therefore not easily made quantitative. The procedure which was finally adopted is based on the observation that insulin, whether in the oxidized or reduced state, is insoluble in 90 per cent acetone and that thioglycolic acid is readily soluble

in this solvent. The samples of the reduction mixture are withdrawn into centrifuge tubes and 9 times their volume of pure acetone added. The contents are thoroughly mixed and centrifuged. The precipitate is washed six times with acetone, three times with ether, and then dried *in vacuo* over sulfuric acid. The accuracy of the sampling is checked by weighing the precipitate. This procedure will work satisfactorily if the reduction mixture has a pH of 2 or less. The acid phthalate used as buffer remains dissolved in the acetone-water mixture. At pH  $>2$  the protein often assumes a gummy consistency when treated with acetone. It is necessary to add some hydrochloric acid to the acetone if insulin solutions of pH  $>2$  are used.

Control experiments have demonstrated that the protein does not suffer appreciable denaturation by the acetone-ether procedure if the process is carried out within 1 hour. Under these conditions it is not necessary to work in a cold room. The thioglycolic acid is quantitatively removed. The sensitive color test with iron salts is negative, and there is no appreciable odor of thioglycolic acid. *If the acetone treatment is carried out immediately after adding the reducer so that no reduction of the protein occurs, a completely soluble protein preparation is obtained which gives a negative —SH test with nitroprusside.* With increasing reduction time the nitroprusside test gradually becomes stronger. If the reduction is carried beyond 150 minutes, an increasing fraction of the protein becomes insoluble in water, indicating denaturation.

### Methods

Three methods were used for the quantitative determination of the thiol groups in reduced insulin. These are all based on the reduction of phosphotungstic acid (Folin-Marenzi reagent (13)) by sulfhydryl compounds. The amount of the blue reduction product formed is determined by photometric analysis. In the first method, use is made of the observation that reduced insulin will react with the phosphotungstic acid reagent directly. This method is called the *direct method*. The second method is based on the principle that the reduced protein will react with an excess of cystine; the protein is reoxidized to the disulfide form and an equivalent amount of cysteine is formed. This principle has been elaborated by Mirsky and Anson (6, 7). In the present paper,

this method is called the *indirect method*, although Mirsky and Anson speak of it as a direct method. The third method consists in the acid hydrolysis of reduced insulin and estimation of the cysteine content of the hydrolysate. This method has recently been applied to a number of proteins by Mirsky and Anson (6, 7).

*Photoelectric Colorimetry*—The intensity of the blue color produced in the reaction of thiols with phosphotungstate is not constant with time. Depending on the composition of the analytical mixtures, the color intensity may either increase or decrease with time. This renders colorimetry by a subjective method unreliable and difficult. Consequently, photoelectric analysis was selected. The instrument used was similar in construction to those described recently by Strafford (14) and by Diller (15). The voltage generated by illumination of the photoelectric cell is balanced against an external source of current by means of a potentiometer.

*Calibration of Colorimeter*—Pure cysteine hydrochloride was used for calibrating the photoelectric colorimeter. The determinations were conducted as described in the following section.

*Direct Method*—8 to 20 mg. of reduced insulin are accurately weighed and dissolved in 5 cc. of water. 4 cc. of 3.4 M phosphate buffer (pH 6.0) are added and the volume made to 25 cc. with urea solution. The addition of the buffer to the protein solution may cause a turbidity. However, the contents of the flask become clear on addition of the urea solution. If the reduction of the insulin has been carried beyond 150 minutes, a fraction of the protein will become insoluble in water. This fraction is dissolved by the urea. Prolonged standing of the mixture should be avoided because urea will slowly denature proteins. If necessary, the contents of the flask are filtered. The blue color is measured in the photometer. Readings are made 5, 10, and 15 minutes after the addition of the Folin-Marenzi reagent. The reading corresponding to the highest extinction (*i.e.* lowest photoelectric potential) is selected for calculation. Usually, the readings after 5 and 10 minutes agree within 1 to 2 per cent.

*Indirect Method*—Approximately 30 mg. of reduced insulin are accurately weighed and dissolved in 5 cc. of water. The solution is placed in the main chamber of the reduction apparatus (Fig. 1), and nitrogen gas passed through it for 30 minutes at 30°. A solution

of 200 mg. of cystine in 3 cc. of 0.5 N KOH is prepared and freed from air by vigorous boiling *in vacuo* at about 35° in a Thunberg tube. The air-free solution is then added to the insulin solution, followed by a drop of caprylic alcohol to prevent frothing. The flow of nitrogen is maintained; after 1 hour the mixture is delivered into a 50 cc. centrifuge tube containing 2 cc. of 10 per cent sodium tungstate solution. The reduction chamber is rinsed twice with 2 cc. of water. The subsequent steps in the analysis are those described by Mirsky and Anson (6). *It is essential to remove all oxygen from the reactants before mixing and to prevent any exposure to air by continuing the flow of nitrogen during the reduction.*

**Hydrolysis Method**—80 to 100 mg. of reduced insulin were used for each hydrolysis. The procedure of analysis was that given by Mirsky and Anson (6), except that the color was developed in a system containing 5 cc. of 0.5 N KOH. Whenever sufficient material was available, duplicate hydrolysis experiments were carried out on each batch of reduced insulin. The —SH analysis was always performed in duplicate on aliquots of each hydrolysate. In the analysis of comparatively large batches of insulin which had been reduced for various lengths of time, all three methods of analysis given above were used.

### *Analysis of Reduced Insulin*

**Course of Reduction**—Of the three methods for —SH determination which have been described, the direct method is the simplest procedure and requires the smallest amounts of insulin. This technique was therefore chosen for a study of the course of reduction of insulin by thioglycolic acid. The variables were hydrogen ion concentration, time of reduction, and temperature. 100 or 200 mg. of insulin were dissolved in water. Buffer solution was added, and the volume made to 10 cc. with water. In most of the experiments acid phthalate-HCl buffer was employed; the final buffer strength was 0.08 M. The technique of reduction was that described above. The pH of the insulin-thioglycolic acid mixtures was measured with the glass electrode. The authors are indebted to Mr. D. DuBois for these measurements. Samples of 1 cc. volume each were withdrawn at various time intervals and the thioglycolic acid removed as already described. The intervals varied from 15 minutes to 60 minutes. In some experiments the

course of reduction was followed for 7 hours. The range from 25 to 200 minutes was carefully investigated. The pH of the reduction mixtures in most of the experiments was between 1.80 and 1.90. One reduction curve was obtained at pH 2.08. Experiments at higher pH values were not satisfactory because of the difficulties, already mentioned, encountered in the acetone-ether procedure, and also because the insulin was partially precipitated during the reduction. In the alkaline range, in which insulin is again soluble, the course of the reaction is complex because the alkali is competing with the reducing agent in the inactivation process. The study of the effect of thioglycolic acid was therefore restricted to the acid range in which insulin is soluble and stable.

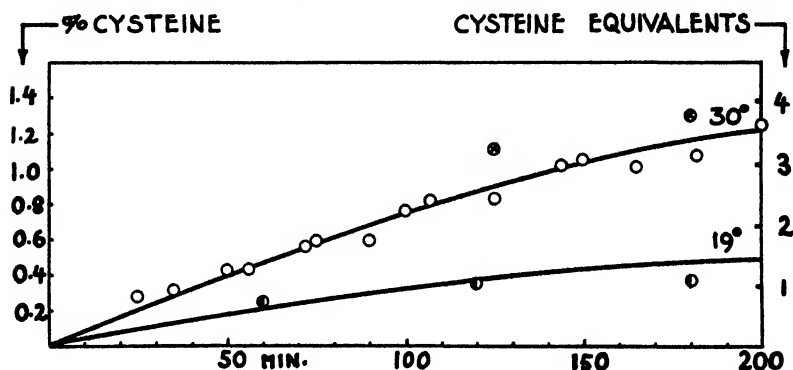


FIG. 2. Course of reduction of insulin by thioglycolic acid at pH 1.8. ○ and ⊗ show results on amorphous insulin; ⊗, results on crystalline insulin.

All of these experiments were conducted at 30°. In addition, one experiment was carried out at 19° in order to determine the temperature coefficient of the reduction. Some of the results obtained are plotted in Fig. 2.

The results are expressed in terms of *cysteine* because cysteine served as the standard in calibrating the photometer and because, as is shown in the following section, there is general agreement among the data obtained by the direct method and those obtained by the indirect and the hydrolysis methods. However, it is realized that a more specific and direct type of evidence is required to prove that it is cysteine which is formed by the reduction of insulin and not a thiol of a similar reduction intensity toward

phosphotungstate. Fig. 2 shows that up to approximately 150 minutes, the relationship between reduction time and the quantity of —SH groups liberated is almost linear. At a later time the slope of the curve decreases. The flattening of the reduction

TABLE I  
*Results of —SH Analysis of Reduced Insulin*

| Reduced insulin sample No. | Reduction time | Temperature | "Cysteine"    |                 |                   |
|----------------------------|----------------|-------------|---------------|-----------------|-------------------|
|                            |                |             | Direct method | Indirect method | Hydrolysis method |
|                            | min.           | °C.         | per cent      | per cent        | per cent          |
| 3                          | 5760           | 23          |               |                 | 1.84*             |
|                            |                |             |               |                 | 1.89*             |
| 4                          | 300            | 30          |               | 0.96†           | 1.42*             |
|                            |                |             |               | 0.93†           | 2.01*             |
|                            |                |             |               | 1.08†           | 1.58              |
|                            |                |             |               | 1.08†           | 1.08              |
| 5                          | 300            | 30          | 1.36          | 1.38†           | 1.35              |
|                            |                |             |               | 1.63†           | 1.21              |
| 6                          | 150            | 30          | 1.20          | 0.91†           | 0.70              |
|                            |                |             | 1.13          | 0.81†           | 0.76              |
|                            |                |             |               | 1.12†           | 1.06‡             |
| 7                          | 180            | 30          | 1.41          | 0.89†           | 1.20‡             |
|                            |                |             | 1.39          | 1.46†           | 1.29‡             |
|                            |                |             |               | 0.75†           | 0.57              |
|                            |                |             |               | 1.50†           |                   |
|                            |                |             |               | 1.14†           |                   |
| 8                          | 180            | 30          | 1.13          | 1.21§           | 1.04‡             |
|                            |                |             | 1.09          | 1.12§           | 1.04‡             |
| 9                          | 150            | 30          | 0.96          | 1.27§           | 0.89‡             |
|                            |                |             | 1.03          | 0.94§           | 0.95‡             |
|                            |                |             |               | 0.99§           |                   |

\* These values were obtained by visual colorimetry. All other values were obtained by photoelectric colorimetry.

† Technique recommended by Mirsky and Anson (6).

‡ KOH was added in the analysis.

§ Improved technique with the reduction apparatus.

curves coincides with the formation of increasing quantities of water-insoluble protein. The belief that this is due to incipient denaturation finds support in the results of the viscosity measurements reported later in this paper. It is interesting to note that



after 200 minutes reduction time (at 30° and pH 1.8) somewhat over 3 cysteine equivalents per molecule of insulin<sup>a</sup> have been formed. An inspection of the reduction curves obtained at 30° and at 19° demonstrates that the reduction of insulin by thioglycolic acid is a chemical reaction with a considerable temperature coefficient.

*Analysis by Three Methods*—300 to 500 mg. of insulin were dissolved in water. Acid phthalate buffer (pH 2.8) and water were added to make the desired volume, which in most cases was 10 cc. Thioglycolic acid in an amount corresponding to 3.5 times the weight of the insulin was added. The final pH in the reduction mixtures was 1.65, measured with the glass electrode. The reduction was carried out at 30° in the apparatus (Fig. 1); it was interrupted after 150, 180, or 300 minutes, and the insulin was obtained free from the reducer by the acetone-ether process. The yield of dry, reduced insulin was 80 to 90 per cent of the starting material. In one experiment, the reduction was conducted for 96 hours at room temperature (about 23°). In this case, the reduction mixture was stored in nitrogen-filled Thunberg tubes under paraffin seals. The results of the analyses of some of these samples are summarized in Table I. Some of the earlier analyses carried out with unsatisfactory techniques have been included in Table I in order to illustrate the points mentioned in the sections dealing with the analytical methods. Although the ratio of insulin to reducer was the same in all the experiments tabulated, the absolute concentrations of both reactants were smaller in Preparations 8 and 9 as compared with the others.

It is seen from Table I that the highest cysteine value found was 2 per cent, corresponding to less than 10 per cent of the total insulin sulfur. Most of the analytical values, however, are nearer to 1 per cent cysteine. The present study is therefore limited to the initial stages of reduction.

#### *Viscosity Changes during Reduction*

When insulin is subjected to the prolonged action of thioglycolic acid at pH 2 or lower, a turbid and rather stiff gel ultimately results. The insulin concentration in mixtures showing this

<sup>a</sup> The molecular weight of insulin between pH 4.5 and pH 7.0 is taken as 35,000 (16).

phenomenon varied from  $3 \times 10^{-4}$  to  $1.4 \times 10^{-3}$  M. Gel formation under these conditions occurs after 7 to 12 hours standing at  $30^\circ$  and after approximately 24 hours at  $20^\circ$ . There is no great

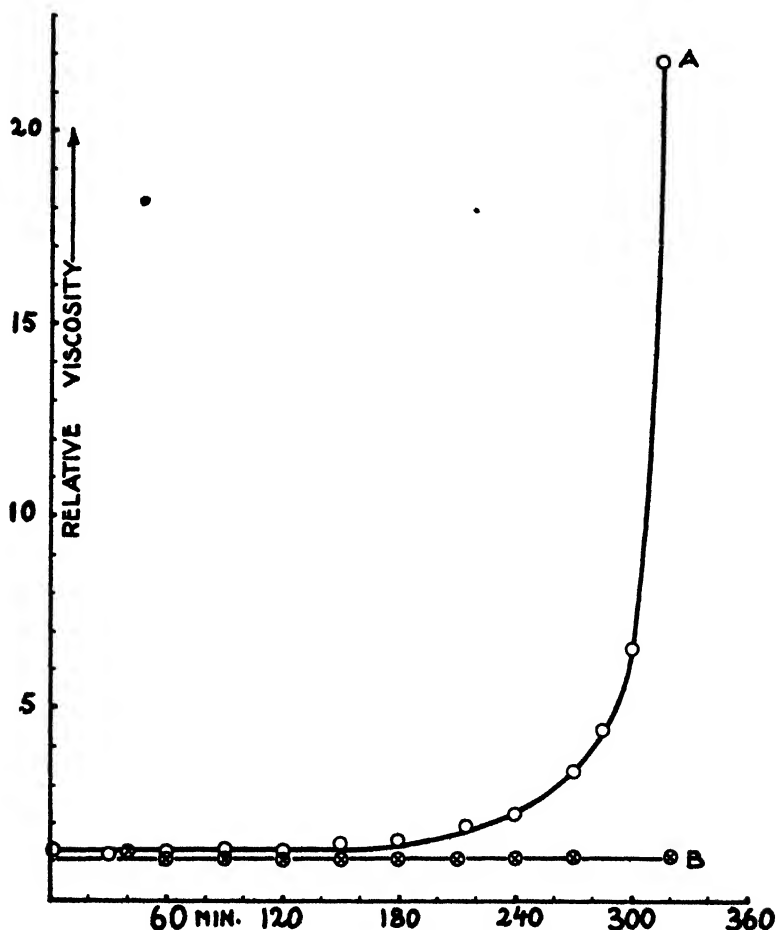


FIG. 3. Curve A shows the viscosity changes during reduction of insulin by thioglycolic acid; Curve B shows the results of a control experiment, without thioglycolic acid.

difference between samples which have been standing under nitrogen gas or those through which nitrogen has been continuously passed. The changes in viscosity during the reduction have been

followed by using the sampling device of the reduction apparatus as a viscosimeter.<sup>4</sup> The reduction mixture is subjected to a constant nitrogen pressure and the time required for the solution to pass the two marks on the burette (see Fig. 1) is recorded. In these experiments it was found necessary to omit the preliminary nitrogen period, because thioglycolic acid is required for the adjustment of the final pH of the mixture. Thereby, the use of caprylic alcohol to prevent frothing is made unnecessary. In Fig. 3 are plotted the results of an experiment in which the viscosity changes at pH 1.88 and 30° were followed for 315 minutes. The insulin concentration was  $6 \times 10^{-4}$  M; the thioglycolic acid concentration was  $8 \times 10^{-2}$  M. The results of a control experiment, in which the reducing agent was omitted, and the pH of the insulin solution adjusted to that of the reduction mixture by means of the glass electrode, are also shown in Fig. 3. In the latter experiment no changes in viscosity could be detected. In the presence of thioglycolic acid the viscosity remained approximately constant only for the first 150 minutes; there followed a rapid increase in viscosity. After 300 minutes the mixture had become so viscous that the nitrogen over-pressure corresponding to 38 cm. of water was unable to force the solution through the capillary of the viscosimeter.

#### *Physiological Activity of Reduced Insulin*

It has been repeatedly asserted that cysteine, glutathione, thioglycolic acid, hydrogen sulfide, and other reducing agents will abolish the pharmacodynamic action of insulin (1, 5). In the majority of these reports, the reduction of the hormone was tested for qualitatively by the nitroprusside test. Wintersteiner (9) measured the decrease in cysteine which he used as a reducer and postulated corresponding conversion of oxidized into reduced insulin. The techniques described in the preceding sections make it possible to follow the course of reduction of insulin in a quantitative manner. The viscosity determinations permit the detection of incipient denaturation or, at least, of marked physical changes. Under the conditions chosen, the latter become manifest after 150 minutes reduction. As long as the protein is soluble in water and retains its original viscosity, it may be considered to be in the

<sup>4</sup> This use was suggested by Dr. L. F. Nims.

native state. The transition to denaturation, marked by decreasing solubility and increasing viscosity of the protein, is quite gradual. After approximately 150 minutes reduction, the protein contains about 1 per cent cysteine (see Table I and Fig. 2), corresponding to the reduction of about 5 per cent of the total sulfur. It was of interest to determine the physiological activity of this reduced, but not yet appreciably denatured, insulin, and also that of other reduced fractions. The bioassay was carried out in the Lilly Research Laboratories under the supervision of Mr. George B. Walden. The mouse convulsion method of assay was used. The results are given in Table II. Each test was conducted on a total of 240 mice on the sample and 240 mice on the standard, with the exception of Sample 7. The latter was assayed on a consider-

TABLE II  
*Physiological Activity of Reduced Insulin*

The original —S—S— insulin had an activity of 20 to 22 units per mg.

| Sample No. | Time of reduction<br>(at 30°) | Physiological activity      |
|------------|-------------------------------|-----------------------------|
|            | min.                          | international units per mg. |
| 10-f       | 150                           | 12.5                        |
| 9          | 150                           | 11.0                        |
| 7          | 180                           | 10.0-11.0                   |
| 8          | 180                           | 10.0-11.0                   |

ably smaller number of animals. The data show that there is preserved in insulin, after 150 minutes reduction, approximately 50 per cent of the original activity.<sup>5</sup> No appreciable change in the activity seems to occur if the reduction is extended to 180 minutes. At this point, the material shows increased viscosity and markedly decreased solubility. Since the physiological activity of any insoluble material present cannot be evaluated by the method of assay used, it was not deemed advisable to proceed further with the reduction. It is obvious, however, that under the

<sup>5</sup> For the bioassay, the insulin preparations were sent in the dry state to Indianapolis. Control experiments have shown that such samples retain their degree of reduction for periods similar to those which elapsed between their preparation and bioassay. Furthermore, unpublished data demonstrate that even in solutions of widely varying pH values, these purified, reduced insulin preparations are not easily autoxidized.

conditions of reduction chosen, it would be necessary to proceed well into the range of denaturation before a complete loss of the physiological activity could be expected.

Inasmuch as a small portion of the 150 minute reduction sample and a considerable quantity of the 180 minute reduction sample were difficultly soluble in water, the values obtained in the bio-assay should be considered as minimum figures. This is particularly true in view of the recent publications on protamine insulinate which stress the important influence of solubility, route of administration, and rate of absorption on the type of physiological response obtained.

#### DISCUSSION

In accordance with the views of Mirsky and Anson, four states of oxidation-reduction in proteins may be listed:

$$\text{Native protein} \begin{cases} \text{Oxidized (I)} \\ \text{Reduced (II)} \end{cases} \quad \text{Denatured protein} \begin{cases} \text{Oxidized (III)} \\ \text{Reduced (IV)} \end{cases}$$

Most proteins occur in nature in State I. In some proteins, *e.g.* hemoglobin, a shift of the pH toward the alkaline range will suffice to shift the equilibrium toward State II. In other proteins, State II is obtained by the action of reducing agents. The number of —SH groups which may so be produced is only a fraction of the total "potential" —SH groups. When a denatured protein is fully reduced, the number of free —SH groups is equal to the total number which can be obtained by hydrolysis of the preparation.

The present experiments indicate that insulin conforms with this scheme. In the native and fully active hormone no free —SH groups are detectable. It is therefore in State I. When insulin is acted upon by thioglycolic acid at pH near 2, the first phase is the conversion to State II. Later in the process the protein suffers marked physical changes, *e.g.* decrease in solubility and increase in viscosity and gel formation, thus indicating denaturation. The rate of appearance of free sulfhydryl groups at this stage decreases (see Fig. 2). By combining chemical and physical methods it is possible to determine the point on the reduction curve at which the hormone exists more or less in State II. At any time, the reduction process may be discontinued and a dry, stable

product, free from the reducing agent, prepared. The results of —SH analysis by three methods indicate that after 150 minutes reduction (probably corresponding to State II), 2 to 3 cysteine equivalents per insulin molecule are present (see Table I). The bioassay of such preparations shows that they have retained at least 50 per cent of the original physiological activity. Complete inactivation would then require more drastic changes in the molecule, possibly leading to complete denaturation.

The results obtained in this investigation would suggest the working hypothesis that one or two dithio ( $\text{—S—S—}$ ) linkages in insulin have a special function. Bersin (17) has recently discussed the possibility that the sulfur-containing groups in certain biocatalysts may not be identical with their *active centers* which react with the substrate, but that they may be *activating groups*, according to the terminology proposed by Langenbeck (18). The applicability of this hypothesis to insulin is under investigation.

The authors wish to record their sincere appreciation of the cooperation of Eli Lilly and Company, through Dr. G. H. A. Clowes, by providing 45 gm. of insulin and technical assistance, and to Mr. G. B. Walden for conducting the bioassay in the Lilly Research Laboratories.

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## THE CHEMISTRY OF THE LACTOGENIC HORMONE EXTRACTS\*

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The chemical investigations which have been made on the anterior pituitary hormones have been chiefly concerned with the isolation of as active and as pure extracts as was possible in the light of the knowledge of these substances. A chemical study of certain of the more highly purified extracts should provide information for use in the further purification and isolation of the anterior pituitary hormones. It was with this aim in view, therefore, that this investigation of the chemistry of the lactogenic hormone extracts was undertaken in order to determine the effects of certain chemical conditions on the activity of the hormone and to obtain information as to the chemical groups responsible for the physiological activity of the hormone.

### EXPERIMENTAL

The lactogenic hormone extracts used in this investigation were prepared by a modified acetic acid (1) and by the acid acetone (2) methods of extraction. These extracts assayed 1 bird unit per mg. according to the method (3) based upon the initiation of proliferation in the crop gland of the pigeon. Other less active, together with certain inactive, extracts were compared with the active extracts.

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The data presented in this paper formed part of a thesis presented by W. H. McShan in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of the University of Missouri, 1936.



The hormone extracts were protein in nature, as indicated by the biuret test. According to the Hopkins-Cole, Millon, and xanthoproteic tests the protein complex consisted in part of tryptophane and tyrosine. The ninhydrin test for the free  $\alpha$ -amino-carboxyl group was positive for the active acetic acid extract and negative for the inactive and acid acetone extracts. The filtrate obtained from the sodium fusion of the hormone material when tested by the usual methods showed the presence of nitrogen and sulfur and the absence of halogen. The phosphorus was detected colorimetrically.

Digestion of the hormone extracts in a series of aliphatic and aromatic solvents for 2 hours at room temperature showed that they were soluble in glacial acetic acid, formamide, 66 per cent aqueous acid acetone, and 70 per cent aqueous alkaline alcohol, while they were partially soluble in 90 per cent phenol and lactic acid. According to Widmark (4) the solubility in glacial acetic acid, formamide, phenol, and methyl alcohol is indicative of proteoses. Even though the lactogenic hormone extracts were soluble in certain of these solvents, they cannot be classed as proteoses, since they were insoluble in water.

Our extracts showed a gradual loss in potency in the presence of a small amount of acetic acid. However, the extracts remained active when the dehydration was extended sufficiently to eliminate the acid and they were kept dry at room temperature.

Gardner (5) found that his extracts were rapidly inactivated on heating in slightly alkaline solution, while Bates and Riddle (6) reported that the potency of their extracts remained intact when heated at 100° for 1 hour and at pH 8.0. The hormone extracts used in this study remained active when heated at 37° at pH 7.4, and at 100° for 1 hour at pH 7.8, while inactivation occurred when they were heated at 100° for 1 hour at pH 3.2 and 9.7. These results indicate that the extracts were relatively stable to heat from approximately pH 7.0 to 8.0.

The extracts were inactivated on heating in boiling 0.1 N sodium carbonate solution for 45 minutes without the detectable loss of any labile sulfur. This inactivation may be due to a reaction which involves the sulfur as it was released on more drastic alkaline treatment. It was also thought that the inactivation by heating in dilute alkali might be due to the loss of free amino nitrogen.

However, this assumption was found to be not true, as the treated extracts contained 0.861 per cent, while the untreated extracts contained 0.853 per cent amino nitrogen.

Inactivation also occurred when the extracts were heated in 0.033 N sodium hydroxide solution at 37° for 4 hours and on heating in 0.1 N hydrochloric acid solution at 100° for 1 hour. The initiation of proliferation in the crop gland of the pigeon was used to determine the activity of the heat-treated extracts.

The activity of the extracts was determined after digestion with solutions of sodium nitrite, sodium cyanide, benzoyl chloride, formaldehyde, and acetic anhydride for 2 hours at room temperature. The material was insoluble in the sodium nitrite solution which turned yellow in color, while it was partially soluble in each of the other digests. The treated material was recovered and administered in amounts equivalent to 10 units of the original material. The acetic anhydride had little effect on the activity, while no definite proliferation was obtained on injection of the extracts which had been subjected to the other reagents.

Benzoyl chloride, formaldehyde, and nitrous acid are known to react with the amino group, but they are not specific for this group, as they may react with other groups such as the imino group. It is probable that the inactivation by these reagents was due to their reaction with some group which is necessary for the material to retain its activity. It is recognized, however, that the inactivation could be due to an unknown reaction such as denaturation.

*Diazotization*—When proteins are diazotized, they often react in alkaline solution with aromatic hydroxyl and amine compounds to form colored substances. This reaction of proteins is not well understood, but Eagle (7) recently suggested that the nitrous acid reacts with the indole group of tryptophane to form a nitrosamine.

The diazotization of the protein associated with the lactogenic hormone extract was carried out by treating 5 cc. of a 4 per cent solution of the hormone extract with 0.8 cc. of 4 N sodium nitrite solution and 4 cc. of 1 N hydrochloric acid. The solution was incubated at 0° for 12 hours. When 1 cc. of the diazotized solution was diluted to 8 cc. and 3 drops of an alkaline solution of  $\alpha$ -naphthol were added, a brilliant red color developed. If the assumption that the diazotization of protein depends upon the imino group of tryptophane is correct, it is definite proof that the

protein associated with the lactogenic hormone contains tryptophane. It is suggestive that this group plays a part in the activity, as the diazotized extracts were inactive and non-toxic when administered to the birds (Table I).

*Enzyme Digestion*—Pfanstiehl's 1:75 trypsin powder and Merck's u.s.p. pepsin powder were used (1 cc. of 2 per cent solution) in the digestion of 50 mg. of the hormone extracts. The experiments were run at 37° for 1, 2, 3, and 4 hours at the optimum pH for the activity of the enzyme. The digestion was almost

TABLE I  
*Chemical Properties of Lactogenic Hormone Extracts \**

| Reagent used                                | Temper-<br>ature | Time | pH  | Stability of extract<br>(crop gland test) |
|---|------------------|------|-----|---|
|   | °C.              | hrs. |     |   |
| Water solution of extract .....             | 37               | 1 0  | 7.4 | Proliferation                             |
| “ “ “ “ .....                               | 100              | 1.0  | 7.8 | “   |
| “ “ “ “ .....                               | 100              | 1.0  | 3.2 | Inactivation                              |
| “ “ “ “ .....                               | 100              | 1.0  | 9.7 | “   |
| 0.1 N sodium carbonate solution..           | 100              | 0.75 |     | “   |
| 0.033 N sodium hydroxide solu-<br>tion..... | 37               | 4.0  |     | “   |
| Sodium nitrite.....                         | 25               | 2.0  |     | “   |
| Benzoyl chloride.....                       | 25               | 2.0  |     | “   |
| Sodium cyanide.....                         | 25               | 2.0  |     | “   |
| Formaldehyde.....                           | 25               | 2.0  |     | “   |
| Acetic anhydride.....                       | 25               | 2.0  |     | Proliferation                             |
| Diazotization.....                          | 0                | 12.0 |     | Inactivation                              |
| Trypsin digestion.....                      | 37               | 2.0  |     | “   |
| Pepsin “ .....                              | 37               | 2.0  |     | “   |

\* These tests apply to both acetic acid and acid acetone extracts.

complete in 1 hour. The digests became light yellow in color and a sulfide odor developed. Blank determinations were made without the use of the enzyme.

The ninhydrin and nitroprusside tests were negative for the blank trypsin digests and the biuret test was positive, while for the digested material the ninhydrin and biuret tests were positive and the nitroprusside test was negative. The amino nitrogen content of the extract was increased from 0.853 per cent to 10.72 per cent. The material could not be precipitated isoelectrically after the

pepsin and trypsin digestions. It may be concluded that the protein associated with the lactogenic hormone is attacked by both trypsin and pepsin, which results in complete inactivation.

*Dialysis*—2 per cent solutions of the lactogenic hormone extracts were dialyzed against distilled water by the use of thin collodion sacks prepared by the method of Eggerth (8). The solution of the material was made by arranging the pH on the alkaline side of the isoelectric point. Precipitation occurred during the dialysis. The active material did not pass through the membrane, as there was no significant difference in the activity of the dialyzed and undialyzed extracts, and no stimulation was obtained on injecting the liquid which surrounded the membrane during the dialysis.

*Nitrogen Content*—The total nitrogen content of the acetic acid and acid acetone extracts which were dried at 100° for 2 hours was 15 per cent as determined by the semimicro-Kjeldahl method. The maximum variation was 0.05 per cent. The inactive acetic acid extracts which were prepared 6 months prior to the active extracts contained 14.40 per cent nitrogen. Within this period of time the procedure for the preparation of the extracts was modified as to the method of extraction and dehydration, which, no doubt accounts in part for this difference in the nitrogen content. Both the air-dried active and inactive extracts contained 13.70 per cent nitrogen.

The free amino nitrogen content of the acid acetone Extract GB (9) which assayed 1 bird unit per mg. was found to be 0.853 per cent. On heating the extract in 0.1 N sodium carbonate solution for 45 minutes inactivation occurred without the loss of free amino nitrogen, as the treated material contained 0.861 per cent. On inactivation by digesting with trypsin the free amino nitrogen increased from 0.853 per cent to 10.72 per cent. The maximum variation was 0.04 per cent for the extracts and 0.1 per cent for the trypsin digests.

*Phosphorus Content*—The phosphorus content of the hormone extracts was determined colorimetrically by the method of Briggs (10). The acetic acid extract contained 0.370 per cent and the acid acetone extract contained 0.324 per cent phosphorus.

*Sulfur Content*—The methods developed for the estimation of organic sulfur have not been entirely satisfactory for the quanti-

tative determination of the sulfur content of proteins and biological substances. The method used in this study was a combination of the methods developed by Parr (11) and Toyoda and Kishi (12). It consisted of heating a mixture of 50 mg. of the extracts, 0.5 gm. of finely ground potassium chlorate, and 5 gm. of sodium peroxide in a tightly closed iron crucible in the muffle at 300° for 1.5 hours, after which the mixture was fused. The sulfur was recovered from the fused mass as the sulfate and determined gravimetrically.

The accuracy of the method was tested by determining the sulfur content of a sample of Hammarsten's casein, and an average of 0.780 per cent was obtained from four determinations, while 26.63 per cent was found for cystine from three determinations

TABLE II  
*Summary of Quantitative Determinations on Hormone Extracts*

| Determination for  | Kind of extract |                 |
|--|-----------------|-----------------|
|  | Acetic acid     | Acid acetone    |
|  | <i>per cent</i> | <i>per cent</i> |
| Total nitrogen (heated 100° for 2 hrs.).....               | 14.96           | 15.000          |
| Free amino nitrogen.....                                   |                 | 0.853           |
| “ “ “ (0.1 N Na <sub>2</sub> CO <sub>3</sub> at 100°)..... |                 | 0.861           |
| “ “ “ (trypsin digest).....                                |                 | 10.720          |
| Phosphorus.....  | 0.370           | 0.324           |
| Total sulfur (active extracts).....                        | 1.516           | 1.518           |
| “ “ (inactive extracts).....                               | 1.511           |                 |

which had a range of 0.4 per cent. Theoretically casein contains 0.8 per cent and cystine 26.69 per cent sulfur.

There was a variation in the percentage of sulfur found for the lactogenic hormone extracts with the time of heating. When the partially fused mixture from the muffle heating was brought just to the fusion point, 1.2 per cent sulfur was obtained. When the material was fused for 10 minutes after the muffle heating, 1.5 per cent sulfur was found. However, when the fusion was continued for 20 minutes, there was no increase in the sulfur, which indicates that the total sulfur content of the hormone extracts is 1.5 per cent. There was no significant difference among the sulfur contents of the acetic acid, the acid acetone, and the inactive extracts (Table II).

## SUMMARY

The lactogenic hormone extracts prepared by the acetic acid and acid acetone methods of extraction used in this investigation were protein in nature.

The relation of the physiological activity of the extracts as indicated by crop gland proliferation to chemical treatments such as diazotization, enzyme digestion, heating in dilute acid and alkali solution, and digestion with reagents known to react with certain groups was studied.

The conditions conducive to optimum stability such as dehydration, temperature, and hydrogen ion concentration were determined.

The solubilities of the extracts which were not dialyzable were determined for a series of organic solvents. Quantitative determinations were made for the nitrogen, phosphorus, and sulfur.

No significant difference was found between the lactogenic hormone extracts prepared by the acetic acid and acid acetone methods of extraction.

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## STUDIES ON KETOSIS

### IX. GLYCOGEN FORMATION FROM VARIOUS PURIFIED AND NATURAL FATS\*

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Although there is ample evidence in the literature that glycerol is completely convertible to glucose or glycogen when administered as such, there is no clear cut proof that it follows the same fate when administered as a constituent of neutral fat. Thus, Chambers and Deuel (1) demonstrated the quantitative conversion of ingested glycerol to glucose in the phlorhizinized dog, while Catron and Lewis (2) as well as Shapiro (3) found that it was a ready source of glycogen in the rat. Moreover, Voegtlin, Dunn, and Thompson (4) report that this trihydric alcohol is able to counteract the hypoglycemic convulsions following insulin, while Shapiro noted that it had a strong ketolytic action. However, no one has been able to demonstrate the formation of "extra sugar" from the glycerol moiety of fat fed to phlorhizinized dogs or to show that the deposition of glycogen occurred in the liver as a result of the feeding of natural fats.

In a recent report from this laboratory (5) it was found that tributyrin as well as trivalerin was a good glycogenic agent, although Wesson oil possessed no ability to serve as a source of this polysaccharide. In addition we were unable to demonstrate as great a glycogen formation after the administration of glycerol and sodium butyrate as occurred after the feeding of an equivalent dose of tributyrin.

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The present experiments were undertaken to investigate further any glycogenic action of various purified triglycerides and to determine whether such a glycogenesis could be accounted for exclusively from the glycerol fragment of the ingested fat. The investigation was broadened to include the study of glycogen formation from natural fats of widely varying composition.

#### EXPERIMENTAL

##### *General Procedure*

Male and female rats of 60 to 120 days of age were used. The animals were fasted 48 hours, following which the substance to be tested was fed by stomach tube. Female rats were employed for the tests in which the animals were killed 8 hours after the administration of the fats, while male rats were used in the other tests (17, 24, 42 hours).

In the main group of experiments the purified fats were administered in doses equivalent to 0.065 mole per sq.m. of body surface. Equivalent dosages of the natural fats were calculated from the average saponification numbers. With the exception of trilaurin, all of the fats were fed as such with a minimum quantity of water (0.5 cc.) to remove the last traces of fat from the stomach tube. Because of the high melting point of trilaurin, it was necessary to administer it dissolved in Wesson oil (13 gm. of trilaurin made to 25 cc. with Wesson oil); the mixture was liquid at body temperature.

Control experiments with litter mates were made with each group of experiments. The animals were killed under amytal anesthesia. The technique for glycogen determination was the same as that employed elsewhere (6) with the exception that a single volume of 40 per cent potassium hydroxide equal to the weight of the liver was used for digestion of the tissue. The use of 1 volume prevents layering, which may result in the loss of some glycogen.

In order to determine the extent of absorption of the fats, that remaining in the gastrointestinal tract was ascertained. This procedure was accomplished by passing 60 to 75 cc. of water heated to 75° through the whole gastrointestinal tract (including large intestine and cecum) under sufficient pressure so that the stomach

and intestines were dilated. A blunt hypodermic needle was tied in the esophagus after removal of the gastrointestinal tract and the water was slowly forced from a large syringe. The acidified washings were extracted four times with ether. This extract was dried several days with anhydrous sodium sulfate, after which it was filtered into weighed extraction flasks and the ether was removed. We have shown (5) that oil can be satisfactorily recovered from the gastrointestinal tract immediately after its administration by a similar procedure. The higher values obtained for ether-extractable material in control experiments on fasted animals in the present tests than those reported earlier is probably traceable to the fact that we have employed the whole gastrointestinal tract here, while earlier only the stomach and small intestines were used.

The purified fats were Eastman products, the purity of which we established by the saponification numbers and the specific gravity. The natural fats used were ordinary commercial products. The saponification numbers, iodine numbers, Reichert-Meissl numbers, and specific gravities of these fats were found to be satisfactory.

### *Results*

The experiments in which the purified triglycerides were fed in doses of 0.065 mole per sq.m. of body surface are recorded in Tables I and II. Table I gives a summary of the results of the experiments in which the rats were killed 8 hours after the administration of the fats, and those in which 17 hours elapsed between the period of feeding and the killing of the rats; Table II records the values for the experiments of 24 and 42 hours duration.

With the exception of trilaurin, the fats were practically completely absorbed in 8 hours, whereas identical values on the fat content of the intestinal contents of fat-fed and control rats were noted after 17 hours. The absorption of trilaurin was very slow owing to its solidification in the gastrointestinal tract. Even after 42 hours the absorption amounted to only 80 per cent.

The administration of the triglycerides of the fats having an even number of carbon atoms was followed by a glycogen formation comparable with that of an isomolecular quantity of glycerol,

TABLE I

*Liver Glycogen of Fasting Female (8 Hours) and Male Rats (17 Hours) after Administration of Various Purified Triglycerides and Glycerol in Doses of 0.065 Mole per Sq. M.*

| Substance fed          | Dose<br>per 100<br>sq.cm. | No. of rats | Mean body<br>weight | Liver glycogen |              |             | M.d.<br>P.e.m.d. | Fat absorbed    |                    |                           |
|------------------------|---------------------------|-------------|---------------------|----------------|--------------|-------------|------------------|-----------------|--------------------|---------------------------|
|                        |                           |             |                     | Maxi-<br>mum   | Mini-<br>mum | Mean        |                  | Average<br>fed† | Average in<br>gut‡ | Per cent<br>ab-<br>sorbed |
| Females                |                           |             |                     |                |              |             |                  |                 |                    |                           |
|                        | mg.                       |             | gm.                 | per<br>cent    | per<br>cent  | per<br>cent |                  | mg.             | mg.                |                           |
| Fasting con-<br>trols  |                           | 18          | 139                 | 0.26           | 0.07         | 0.15        |                  |                 | 27.4 (9)           |                           |
| Glycerol con-<br>trols | 59.3                      | 15          | 123                 | 1.62           | 0.15         | 0.83        | 12.37            |                 | 29.2 (7)           |                           |
| Triacetin              | 141                       | 7           | 118                 | 0.86           | 0.32         | 0.59        | 8.00             | 324.2           | 41.8 (5)           | 95.4                      |
| Tripropionin           | 168                       | 9           | 131                 | 2.59           | 0.86         | 1.41        | 10.67            | 386.9           | 33.3 (6)           | 98.4                      |
| Tributylin             | 195                       | 9           | 115                 | 1.58           | 0.27         | 0.68        | 6.78             | 418.6           | 27.5 (6)           | 100                       |
| Trivalerin             | 222                       | 9           | 109                 | 1.58           | 0.64         | 1.16        | 11.48            | 458.6           | 55.8 (7)           | 93.9                      |
| Tricaproin             | 249                       | 9           | 111                 | 0.89           | 0.25         | 0.46        | 5.63             | 531.6           | 31.3 (8)           | 99.2                      |
| Triheptylin            | 277                       | 8           | 104                 | 1.97           | 0.62         | 1.37        | 11.69            | 549.5           | 87.6 (6)           | 89.2                      |
| Tricaprylin            | 304                       | 9           | 138                 | 1.68           | 0.13         | 0.62        | 3.81             | 732.5           | 64.8 (9)           | 94.8                      |
| Trilaurin§             | 402                       | 9           | 140                 | 0.26           | 0.08         | 0.15        |                  | 980.0           | 757.0 (6)          | 25.5                      |
| Males                  |                           |             |                     |                |              |             |                  |                 |                    |                           |
| Fasting con-<br>trols  |                           | 28          |                     | 0.55           | 0.04         | 0.19        |                  |                 | 33.3 (19)          |                           |
| Glycerol con-<br>trols | 59.3                      | 15          | 189                 | 0.83           | 0.04         | 0.31        |                  |                 | 17.4 (3)           |                           |
| Triacetin              | 141                       | 9           | 181                 | 0.70           | 0.06         | 0.26        |                  | 426             | 27.4 (7)           | 100                       |
| Tripropionin           | 168                       | 10          | 200                 | 2.34           | 0.98         | 1.63        | 18.98            | 517             | 24.5 (9)           | 100                       |
| Tributylin             | 195                       | 9           | 192                 | 0.55           | 0.06         | 0.17        |                  | 606             | 27.4 (7)           | 100                       |
| Trivalerin             | 222                       | 10          | 202                 | 2.16           | 0.83         | 1.46        | 12.09            | 673             | 35.0 (7)           | 99.8                      |
| Tricaproin             | 249                       | 12          | 210                 | 0.38           | 0.00         | 0.11        |                  | 762             | 28.2 (8)           | 100                       |
| Triheptylin            | 277                       | 16          | 207                 | 2.98           | 0.43         | 1.44        | 12.27            | 842             | 28.8 (9)           | 100                       |
| Tricaprylin            | 304                       | 9           | 206                 | 0.53           | 0.14         | 0.35        | 7.04             | 943             | 23.3 (6)           | 100                       |
| Trilaurin§             | 402                       | 9           | 213                 | 0.59           | 0.12         | 0.24        |                  | 1315            | 914.1 (8)          | 33.0                      |

\* Ratio of mean difference to probable error of mean difference compared with the glycogen of the controls.

† Average amount fed only for those animals in which the fat content of the gut was analyzed.

‡ The values in parentheses represent the number of experiments on which this analysis was made.

§ Trilaurin was fed dissolved in Wesson oil. The average fed is computed for the trilaurin alone, although approximately an equal amount of Wesson oil was given. The absorption is calculated by considering the fat recovered from the gut as trilaurin.

the values for triacetin, tributyrin, tricaproin, and tricaprylin being 0.59, 0.68, 0.46, and 0.62 per cent compared with a mean of 0.83 for the glycerol controls and one of 0.15 per cent for the fasting controls. All the increases are significant from a statistical standpoint when compared with the level of the fasting control. Trilaurin alone fails to elicit a glycogenesis. With the exception of tricaprylin, the glycogen in the liver has returned to the fasting level 17 hours after feeding the triglycerides mentioned above.

TABLE II

*Liver Glycogen of Fasting Male Rats 24 and 42 Hours after Administration of Tripropionin and Trilaurin in Doses of 0.065 Mole per Sq. M.*

|         | Substance fed    | Dose per sq.cm. | No. of rats | Mean body weight | Liver glycogen |          |          | Fat absorbed |                 |                   |
|---------|------------------|-----------------|-------------|------------------|----------------|----------|----------|--------------|-----------------|-------------------|
|         |                  |                 |             |                  | Maximum        | Minimum  | Mean     | Average fed* | Average in gut† | Per cent absorbed |
|         |                  | mg.             |             | gm.              | per cent       | per cent | per cent | mg.          | mg.             |                   |
| 24 hrs. | Fasting controls |                 | 5           |                  | 0.28           | 0.05     | 0.17     |              | 28.9 (4)        |                   |
|         | Tripropionin     | 168             | 5           | 214              | 2.12           | 0.24     | 1.17     | 532          | 57.0 (3)        | 94.7              |
|         | Trilaurin        | 402             | 4           | 196              | 0.29           | 0.07     | 0.16     | 1222         | 244.3 (3)       | 82.3              |
| 42 hrs. | Fasting controls |                 | 5           |                  | 0.82           | 0.07     | 0.30     |              | 34.9 (5)        |                   |
|         | Tripropionin     | 168             | 4           | 219              | 0.80           | 0.11     | 0.44     | 548          | 30.4 (4)        | 100               |
|         | Trilaurin‡       | 402             | 5           | 203              | 0.49           | 0.09     | 0.30     | 1206         | 296.4 (4)       | 78.4              |

\* Average amount fed only for those animals in which the fat content of the gut was analyzed.

† The values in parentheses represent the number of experiments on which this analysis was made.

‡ Trilaurin was fed dissolved in Wesson oil. The average fed is computed for the trilaurin alone, although approximately an equal amount of Wesson oil was given. The absorption is calculated by considering the fat recovered from the gut as trilaurin.

A much greater increase in liver glycogen followed the administration of tripropionin, trivalerin, and triheptylin than could be accounted for on the basis of the glycerol content. The glycogen level is somewhat higher after 17 hours than at the 8 hour interval, the mean values for tripropionin, trivalerin, and triheptylin at the latter interval being 1.63, 1.46, and 1.44 per cent, respectively, compared with a level in the fasted animals of 0.19 per cent. The average glycogen value after tripropionin was still increased over

TABLE III

*Liver Glycogen of Fasting Female Rats (8 Hours) after Administration of Various Fats in Doses Equivalent to 0.065 Mole per Sq. M.*

| Substance fed    | Dose per 100 sq. cm. | No. of rats | Mean body Weight | Liver glycogen |          |          | Fat absorbed |                 |                   |
|------------------|----------------------|-------------|------------------|----------------|----------|----------|--------------|-----------------|-------------------|
|                  |                      |             |                  | Maximum        | Minimum  | Mean     | Average fed* | Average in gut† | Per cent absorbed |
|                  | mg.                  |             | gm.              | per cent       | per cent | per cent | mg.          | mg.             |                   |
| Fasting controls |                      | 38          | 140              | 0.26           | 0.06     | 0.12     |              | 25.4(24)        |                   |
| Cottonseed oil   | 759                  | 10          | 154              | 0.09           | 0.06     | 0.08     | 1930         | 742.1(8)        | 62.9              |
| Wesson oil       | 761                  | 18          | 121              | 0.38           | 0.03     | 0.13     | 1796         | 684.0(6)        | 63.3              |
| Linseed "        | 777                  | 10          | 146              | 0.15           | 0.10     | 0.13     | 1938         | 767.8(8)        | 61.7              |
| Peanut "         | 780                  | 10          | 147              | 0.66           | 0.06     | 0.14     | 1972         | 762.8(9)        | 62.6              |
| Coconut "        | 586                  | 10          | 151              | 0.22           | 0.12     | 0.18     | 1462         | 540.3(7)        | 64.7              |
| Butter fat       | 675                  | 10          | 145              | 0.19           | 0.06     | 0.09     | 1680         | 443.3(7)        | 75.2              |

\* Average amount fed only for those animals in which the fat content of the gut was analyzed.

† The values in parentheses represent the number of experiments on which this analysis was made.

TABLE IV

*Liver Glycogen of Fasting Male Rats 17 Hours after Administration of Various Fats in Doses Equivalent to 0.065 Mole per Sq. M.*

| Substance fed    | Dose per 100 sq. cm. | No. of rats | Mean body weight | Liver glycogen |          |          | M.d. P.e.m.d. | Fat absorbed |                 |                   |
|------------------|----------------------|-------------|------------------|----------------|----------|----------|---------------|--------------|-----------------|-------------------|
|                  |                      |             |                  | Maximum        | Minimum  | Mean     |               | Average fed† | Average in gut† | Per cent absorbed |
|                  | mg.                  |             | gm.              | per cent       | per cent | per cent |               | mg.          | mg.             |                   |
| Fasting controls |                      | 18          |                  | 0.72           | 0.10     | 0.28     |               |              | 36.9(13)        |                   |
| Cottonseed oil   | 759                  | 11          | 272              | 0.29           | 0.05     | 0.20     |               | 2730         | 266.5(11)       | 91.6              |
| Wesson oil       | 761                  | 13          | 204              | 0.62           | 0.05     | 0.22     |               | 2278         | 101.0(10)       | 97.2              |
| Linseed "        | 777                  | 10          | 281              | 0.44           | 0.07     | 0.25     |               | 2965         | 279.9(6)        | 91.8              |
| Peanut "         | 780                  | 10          | 289              | 0.96           | 0.09     | 0.31     |               | 2920         | 171.9(7)        | 95.4              |
| Coconut "        | 586                  | 19          | 221              | 0.70           | 0.12     | 0.39     | 3.06          | 1880         | 74.6(10)        | 97.8              |
| Butter fat       | 675                  | 10          | 273              | 0.72           | 0.06     | 0.34     | 1.07          | 2435         | 185.2(4)        | 93.9              |

For explanation of foot-notes, see Table I.

that of the controls after 24 hours (1.17 per cent) but had practically dropped to the fasting level after 42 hours (0.44 per cent).

In Tables III to V are reported similar experiments on rats with natural fats at like intervals after the fat feeding.

In distinction to the purified fats, the absorption of the natural fats was incomplete in every case at 8 hours. This is probably traceable to the greater bulk fed in the latter case, owing to the increasing molecular weight. In the experiments with 17 hours interval, the absorption exceeded 90 per cent. None of the averages of liver glycogen in the 8 hour test is increased over the level

TABLE V

*Liver Glycogen of Fasting Male Rats 24 and 42 Hours after Administration of Various Fats in Doses of 0.065 Mole per Sq. M.*

|         | Substance fed         | Dose per 100 sq.cm. | No. of rats | Mean body weight | Liver glycogen |              |             | M.d.<br>P.m.d. | Fat absorbed    |                    |                              |
|---------|-----------------------|---------------------|-------------|------------------|----------------|--------------|-------------|----------------|-----------------|--------------------|------------------------------|
|         |                       |                     |             |                  | Maxi-<br>mum   | Mini-<br>mum | Mean        |                | Average<br>fed† | Average<br>in gut‡ | Per<br>cent<br>ab-<br>sorbed |
|         |                       | mg.                 |             | gm.              | per<br>cent    | per<br>cent  | per<br>cent |                | mg.             | mg.                |                              |
| 24 hrs. | Fasting con-<br>trols |                     | 28          | 192              | 0.89           | 0.05         | 0.29        |                |                 | 31.1 (9)           |                              |
|         | Wesson oil            | 761                 | 15          | 188              | 0.57           | 0.06         | 0.30        |                | 2275            | 83.8 (8)           | 97.7                         |
|         | Coconut "             | 586                 | 30          | 203              | 1.08           | 0.08         | 0.37        | 2.00           | 1765            | 53.9 (9)           | 98.8                         |
|         | Butter fat            | 675                 | 29          | 196              | 0.97           | 0.06         | 0.33        | 1.06           | 2365            | 59.0 (7)           | 98.8                         |
| 42 hrs. | Fasting con-<br>trols |                     | 15          |                  | 0.82           | 0.05         | 0.21        |                |                 | 32.5 (10)          |                              |
|         | Wesson oil            | 761                 | 12          | 207              | 0.47           | 0.05         | 0.17        |                | 2364            | 54.3 (9)           | 99.2                         |
|         | Coconut "             | 586                 | 15          | 215              | 0.71           | 0.05         | 0.27        |                | 1806            | 36.8 (10)          | 99.8                         |
|         | Butter fat            | 675                 | 15          | 203              | 0.36           | 0.06         | 0.16        |                | 2385            | 30.6 (9)           | 100                          |

For explanation of foot-notes, see Table I.

of the control animals. On the other hand the values of liver glycogen are slightly but significantly higher 17 hours and 24 hours after the feeding of coconut oil than in the control animals. A similar tendency is noted in the experiments with butter fat. All the other natural fats (Wesson oil, cottonseed oil, peanut oil, linseed oil) are entirely void of glycogenic action.

#### DISCUSSION

All of the purified triglycerides from triacetin through tricapylin are definite sources of glycogen in the fasting rat, while most of the

natural fats such as Wesson oil, cottonseed oil, peanut oil, and linseed oil are entirely without glycogenic ability, although a slight formation of this polysaccharide sometimes followed the administration of similar amounts of coconut oil or of butter fat. The average percentage of glycogen with fats having fatty acids with an odd number of carbon atoms usually approximates 1.50 per cent, although an individual value as high as 2.98 per cent was noted with triheptylin. Even with those fats with acids having an even number of carbon atoms, one notes an individual value as high as 1.58 per cent (tributylin), although the averages are near 0.5 per cent for the single dose.

The glycogen content of the livers of the rats receiving triacetin, tributyrin, tricaproin, and tricaprylin can be accounted for on the basis of the glycerol components of these fats. We now believe that the inability to record as high values in the control experiments with hydrolyzed tributyrin as with the neutral fat which we reported in our earlier work (5) is due to the alkaline effect of the residue resulting from the combustion of sodium butyrate. A glycogenolysis must occur to furnish the lactic acid needed for such a neutralization. There is no evidence that the short chained fatty acids with an even number of carbon atoms contribute to the glycogen so stored in the liver. Not only have we found (5) that the sodium salts of butyric, caproic, and caprylic acids were entirely without potentiality as sources of glycogen but also we have noted (7) that the ethyl esters of these acids are also ineffective. In the latter case any possible criticism that the glyco-genesis is obscured by a possible alkalosis is avoided.

The question arises as to why the glycerol of the lower molecular fats is able to produce glycogen when that from such fats as cottonseed oil, peanut oil, or linseed oil with high molecular weights cannot do so. It has been demonstrated by Eckstein that neither tributyrin (8) nor tricaproin (9) can be stored in the fatty tissues, while Powell (10) has shown the same to be true with tricaprylin. On the other hand tricaprin (11) and trilaurin (8, 10) are both capable of retention in the depot fat, as are the glycerides of the higher acids of which such natural fats as cottonseed, peanut, or linseed oil are almost entirely composed.

The fats which cannot be retained in the tissues either are not resynthesized into the neutral fat after absorption or if resynthesis

does occur, the fat is soon broken down and the fatty acid is either completely oxidized or built into a new longer chain fatty acid. In any event considerable amounts of glycerol are left behind and this is converted into glycogen. In view of the fact that the glycogen level of the liver after triacetin, tributyrin, tricaproin, and tricaprylin approximates that of the glycerol controls, it is suggested that the resynthesis of the short chain acids into longer chain fatty acids (with a new formation of neutral fat) is unimportant and that most of the fatty acid must disappear, leaving the major amount of glycerol free to be transformed into glycogen. Because of the slowness of absorption of trilaurin, the present tests do not give absolute evidence about the fate of this fat. However, the fact that no glycogen whatsoever was noted in the tests as long as 42 hours after the feeding of this triglyceride (at which time the trilaurin was 80 per cent absorbed) would seem to indicate that it was not a glycogen former. This would fit in with the experimental findings that it can be deposited in the tissues (8, 10).

On the other hand, such natural fats as cottonseed, peanut, and linseed oils which can be retained as neutral fats in the tissues have no glycerol available for glycogen synthesis, but it is entirely stored as a component part of the neutral fat. In none of the tests could any glycogen formation be demonstrated after the administration of these neutral fats.

Coconut oil and possibly butter fat apparently give rise to small but definite amounts of glycogen. These fats are the only common ones with an appreciable content of the low weight triglycerides. Although both of these fats contain about the same amount of triglycerides of fatty acids of 8 carbon atoms and less (9.08 per cent for butter fat as compared with 7.20 per cent for coconut oil), coconut oil has an extremely large proportion of tricaprin and trilaurin (66.40 per cent), while butter fat has practically none of these triglycerides (12).

A much higher glycogen level followed the administration of the fats with short chain acids having an odd number of carbon atoms (tripropionin, trivalerin, and triheptylin) than occurred after the corresponding even chained acids. This is further support of our earlier proof that propionic, valeric, and heptonic acids are glycogenic (5) when fed as the sodium salts. The close uniformity in level both at 8 hours (1.41, 1.16, 1.37) and at 17 hours (1.63, 1.46,



1.44) would seem to indicate that tripropionin, trivalerin, and triheptylin are of equal value as glycogen formers.

#### SUMMARY

The administration of triglycerides having fatty acids with an even number of carbon atoms (triacetin, tributyrin, tricaproin, tricaprylin) to fasting rats was followed by the deposition of significant amounts of glycogen in the liver. The source of glycogen could be accounted for on the basis of transformation of the glycerol of the triglycerides.

After the feeding of triglycerides having odd chain fatty acids (tripropionin, trivalerin, triheptylin) to fasting rats, much larger amounts of glycogen were found in the liver than could have originated from the glycerol moiety of the fat. This is further proof of the convertibility of the odd chain fatty acids into carbohydrate.

Trilaurin and a number of natural fats (Wesson oil, cottonseed oil, peanut oil, linseed oil) possessed no glycogenic activity, although significant quantities of glycogen resulted from the ingestion of coconut oil and butter fat.

It is suggested that those fats which cannot be stored as such in the tissues (triglycerides up to and including tricaprylin) are decomposed, with the result that the glycerol is available for glycogen synthesis; on the other hand, the fats which can be deposited in the tissues as such yield no glycogen and the glycerol is stored away as a component of the neutral fat. Coconut oil and possibly butter fat serve as sources of glycogen in proportion to the amount of triglycerides of small molecular weight which they contain.

The triglycerides of the fatty acids through caprylic in doses of 0.065 mole are practically completely absorbed in 8 hours, while the natural fats in similar molecular doses are only about 62 per cent absorbed (butter fat 75 per cent). The latter are completely absorbed in 17 hours.

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## **STUDIES ON KETOSIS**

### **X. GLYCOGEN SYNTHESIS AFTER ETHYL ESTERS OF VARIOUS FATTY ACIDS\***

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Considerable evidence has been brought forward to demonstrate that the fatty acids with an odd number of carbon atoms are glyco-genic, while those with an even number are not. Thus, the sodium salts of propionic, valeric, heptolic, and pelargonic acids gave rise to liver glycogen in the rat (1), while much larger amounts of this polysaccharide were noted in the livers of rats fed tripropionin, trivalerin, or triheptylin than could be ascribed to the glycerol present in the molecule (2). Moreover, it was demonstrated that the fatty acids with an even number of carbon atoms were not glycogen formers when either the sodium soaps or the glycerides were fed.

In view of the fact that we have studied the ketolytic effect of a larger number of the ethyl esters of the fatty acids (3), it was desired to determine whether the non-ketolytic esters were glyco-genic and whether the ketolytic possessed no such ability. Also, because of the ready absorbability of the ethyl esters as compared with the soaps, it has been possible to extend our studies to members having much higher molecular weights than was possible with the sodium salts.

The experimental procedures were similar to that employed in the earlier study (2). C.P. esters from the Eastman Kodak Company were employed. The results are summarized in Table I.

The ethyl esters of the odd carbon fatty acids are all glycogenic,

\* This work was assisted by a research grant from the Rockefeller Foundation.

although the increase over the control level after ethyl undecylate is only slight. Maximum values were usually obtained on the group killed 8 hours after the administration of the ester. The rather low results obtained with ethyl propionate may be ascribed to the toxicity of this ester.

TABLE I

*Liver Glycogen (in Per Cent) of Fasting Female Rats at Various Periods after Administration of Ethyl Esters of Fatty Acids in Doses of 0.088 Mole per Sq.M.*

| Ethyl ester fed   | Liver glycogen at various intervals |        |        |        |        |         |         |                  |
|-------------------|-------------------------------------|--------|--------|--------|--------|---------|---------|------------------|
|                   | Fasted controls                     | 2 hrs. | 4 hrs. | 6 hrs. | 8 hrs. | 10 hrs. | 14 hrs. | Fasted controls* |
| Glycogenic        |                                     |        |        |        |        |         |         |                  |
| Propionate. ....  | 0.16                                | 0.19   | 0.21   | 0.25   | 0.46   | 0.54†   | 0.55    | 0.12             |
| Valerate. ....    | 0.17                                | 0.26   | 0.57   | 0.55   | 0.87   | 0.83    | 0.57    | 0.12             |
| Heptate. ....     | 0.31                                | 0.30   | 0.27   | 0.30   | 0.84   | 0.30‡   | 0.53    | 0.12             |
| Pelargonate. .... | 0.18                                | 0.15   | 0.25   | 0.40   | 0.45   | 0.34    | 0.34    | 0.12             |
| Undecylate. ....  | 0.17                                | 0.15   | 0.26   | 0.14   | 0.22   | 0.25    | 0.27    | 0.12             |
| Non-glycogenic    |                                     |        |        |        |        |         |         |                  |
| Butyrate. ....    | 0.17                                |        |        |        | 0.09   |         |         |                  |
| Caproate. ....    | 0.18                                |        |        |        | 0.17   |         |         |                  |
| Caprylate. ....   | 0.17                                |        |        |        | 0.13   |         |         |                  |
| Caprate. ....     | 0.17                                |        |        |        | 0.10   |         |         |                  |
| Laurate§. ....    | 0.16                                |        |        |        | 0.16   |         |         |                  |
| Myristate. ....   | 0.16                                |        |        |        | 0.13   |         |         |                  |
| Oleate. ....      | 0.16                                |        |        |        | 0.13   |         |         |                  |

Averages of five animals in each group with the following exceptions: eight each in 14 hour tests, as well as second control series; eleven in 8 hour propionate tests.

\* Single group killed at the same period as the 14 hour group.

† One abnormally low group not considered in the average.

‡ Experiments on rats with control level of 0.16 per cent.

§ Methyl laurate used.

On the other hand the liver glycogen of animals fed the esters of the fatty acids with an even number of carbon atoms in every case was as low as that of the fasting controls. That these esters are absorbed and metabolized is proved by our earlier tests in which it has been demonstrated that they give rise to acetone bodies (3). The negative results on glycogen also prove that the ethyl alcohol formed by hydrolysis is not a glycogen former, which

is in harmony with the earlier results of Shapiro (4). Also it is apparent that oleic acid does not break down *in vivo* to azelaic and pelargonic acids; otherwise glycogen rather than the acetone bodies (3) would result.

#### SUMMARY

The ethyl esters of the odd carbon fatty acids (propionic to undecylic) give rise to glycogen when fed to fasting rats, while those of the even carbon fatty acids (butyric to myristic and oleic) give entirely negative results.

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**PHOSPHORIC ACID ESTERS FROM YEAST EXTRACT**  
**THE ISOLATION OF A CRYSTALLINE CALCIUM SALT CONSIST-**  
**ING OF AN EQUIMOLAR MIXTURE OF GLUCOSEMONO-**  
**PHOSPHATE AND GLYCEROPHOSPHATE**

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The original purpose of this investigation was to prepare glucosemonophosphate to serve as a substrate for certain enzymatic studies. This substance has been prepared from yeast by Robison and King (1) and found to have an aldose value (iodometric titration) (2) of 100 per cent. Preparations by other workers have always had a considerably lower aldose value. The usual interpretation given to this is that the preparation of hexosemonophosphate is a mixture of aldose and ketose components. The authority often cited for this interpretation is the widely quoted statement that Robison and King separated such a preparation into these two components. Actually, however, Robison and King (1) state that the analyses of their barium salts indicated the presence of some unknown ester, since the analytical data could not be reconciled with any possible mixture of the then known esters. They further conclude from the Hagedorn-Jensen values that this unknown ester is present in considerable amounts.

All of the earlier work with these compounds suffers from the fact that the preparations studied were not crystalline. At best they were purified over the crystalline alkaloid salts and then reconverted to the non-crystalline barium salts for study. The first non-alkaloid crystalline preparation, so far as the author is aware, is the calcium salt prepared by Warburg and Christian (3). This preparation had an aldose value of about 50 per cent. The calcium content of two fractions was 14.0 and 13.8 per cent. The specific rotation of these same fractions was  $[\alpha]_{546}^{\text{free acid}} = +25.4^{\circ}$



and  $+28.9^{\circ}$  respectively. The compound was readily attacked by their enzyme system and readily fermented by yeast extract.

#### EXPERIMENTAL

The preparation described here was isolated by following the directions given by Warburg and Christian with the following exceptions. The solution of glucose and phosphate was added to the yeast extract continuously from a dropping funnel while the extract was gently shaken by a mechanical shaker. The rate of addition was such as to keep the rate of fermentation, judged by allowing the  $\text{CO}_2$  to bubble out through water, at or near the maximum. With our extracts, prepared from a beer yeast kindly supplied to us by Ruppert's brewery, it is essential that the substrate is not added too rapidly. Otherwise the fermentation is markedly depressed and the yield of the desired product is very small. The rest of the procedure was as Warburg and Christian describe except that the treatment with charcoal was found to be unnecessary. I have carried out this preparation a number of times and have never failed to obtain as the crude product a well crystallized calcium salt in the form of small needles, to a large extent grouped in rosettes.

This product, although well crystallized, can readily be shown to be a mixture. Our attention is limited here to a recrystallized fraction consisting of the first crystals that separate from an aqueous solution of the crude product upon the addition of alcohol at room temperature to a faint turbidity followed by heating.<sup>1</sup> Unless the preceding step—the removal of barium with  $\text{H}_2\text{SO}_4$ —was performed very carefully, this precipitate contains a small amount of  $\text{CaSO}_4$ . This is readily removed by recrystallization. These purified crystals, after drying over  $\text{CaCl}_2$ , have a calcium content of 14.1 per cent, a phosphorus content of 10.4 per cent, a specific rotation (calculated on the basis of 6 carbon atoms per

<sup>1</sup> The amount of this product expressed as percentage of the total yield varies considerably from experiment to experiment. The amount of alcohol required to precipitate it depends upon its concentration and the sharpness of the separation depends upon its relative concentration. If, as is frequently the case, this product makes up 50 per cent or more of the total, and the esters are in solution in from 5 to 10 times their weight of water, a quite pure product is obtained with about 20 per cent alcohol.

phosphorus atom and no water of crystallization) of  $[\alpha]_{546}^{\text{free acid}} = +29.4^{\circ}$ ,<sup>3</sup> and an aldose value (calculated on the basis of one aldose group per phosphorus atom) of 52 per cent. They can be recrystallized repeatedly by dissolving in water, adding alcohol to turbidity, and then heating on a water bath, without any change in composition. A solution of the potassium salt of these crystals is readily oxidized by cytolized red blood cells plus methylene blue, and readily fermented by yeast extract (Fig. 1).

On the basis of the data given these crystals could be considered as a preparation of hexosemonophosphate. However, if such is the case, they must contain about 50 per cent of a non-aldose derivative. Lohmann (4) has developed a method of distinguishing between glucose- and fructosemonophosphate, based on the fact that when heated at  $100^{\circ}$  in 1.0 N acid the fructose ester is hydrolyzed much more rapidly than the glucose. When these crystals are heated in this way with 1.0 N  $\text{H}_2\text{SO}_4$ , it is found that the phosphorus is very resistant to hydrolysis. After 3 hours only 4.5 per cent of the phosphorus has become inorganic. The  $K$  for the hydrolysis, calculated as a first order reaction, is  $0.12 \times 10^{-3}$ , thus agreeing approximately with what Lohmann terms his "rest" ester. Under the same conditions Neuberg ester (fructose-6-phosphate) prepared from carefully purified hexosediphosphate is 27 per cent hydrolyzed in 1 hour. Even at  $115^{\circ}$  these crystals are only 29 per cent hydrolyzed in 3 hours. It thus appears that this preparation contains no appreciable quantity of Neuberg ester. The presence of either the recently described ester (5), believed to be glucose-1-phosphate, or of fructose-1-phosphate (6), can also be ruled out by these figures, since both of these are also readily hydrolyzed. The low aldose value of our preparation must be explained on the basis of some difficultly hydrolyzable ester. In addition to this the Hagedorn-Jensen value is low. Calculated on the assumption that 1 glucose molecule is present per phosphate atom, this value is only 50 per cent that of an equivalent amount of pure glucose, whereas both glucose- and fructosemonophosphates are reported to give about 78 per cent of the glucose value.

The most important point seemed to be to determine whether

<sup>3</sup> Determined by dissolving 34.83 mg. of calcium salt in 0.5 cc. of 1.0 N HCl. The rotation in a 0.5 dm. tube was  $+0.89^{\circ}$ .

or not the preparation really is a hexosemonophosphate, and for this purpose the carbon content seemed to be essential. It is surprising how few carbon analyses on such esters are recorded in the literature. This is probably due to the high ash content, although this is no serious objection when the method of Van Slyke, Page, and Kirk (7) is used. Carbon analyses were carried out on this preparation with both the usual Pregl<sup>3</sup> and the Van Slyke methods. In the Pregl determinations the salt was previously mixed with  $K_2Cr_2O_7$  and with  $CuO$ .

6.405 mg. sample +  $K_2Cr_2O_7$ . 4.395 mg.  $CO_2$ , C 18.71%, 2.510 mg.  $H_2O$ ,  
H 4.38%

6.302 mg. sample +  $CuO$ . 4.216 mg.  $CO_2$ , C 18.25%, 2.425 mg.  $H_2O$ ,  
H 4.31%

The same sample by the Van Slyke method gave

2.530 mg. sample,  $PCO_2$  334.0 mm.,  $T$  23°: C 0.4716 mg., 18.64%  
2.581 " " " 343.1 " " 25°: " 0.4797 " 18.58%

The calcium (de Waard (8)) was 14.2 and 14.0 per cent. The phosphorus (Fiske and Subbarow (9)) was 10.24 and 10.40 per cent. Nitrogen was not present.

|  | C     | H    | P     | Ca    |
|--|-------|------|-------|-------|
| Found.....                                   | 18.55 | 4.3  | 10.32 | 14.1  |
| Calculated for $C_9H_{24}O_{19}P_2Ca_2$ .... | 18.7  | 4.15 | 10.7  | 13.85 |
| " " $C_9H_{11}O_4PCa$ .....                  | 24.2  | 3.7  | 10.4  | 13.4  |

It is evident from these figures that despite the fact that the phosphorus content checks with that of an anhydrous calcium salt of a hexosemonophosphate and despite the fact that the calcium to phosphorus ratio is 1, the substance is not a hexosemonophosphate. The figures agree fairly well with the formula  $C_9H_{24}O_{19}P_2Ca_2$ . This is a 9-carbon diphosphate containing 4 molecules of  $H_2O$  (i.e. 2 of water per calcium). 1 of these water molecules can be removed by prolonged drying over  $P_2O_5$ . The other apparently cannot be removed in this way. Drying over  $H_2SO_4$  must be avoided, for it causes the otherwise pure white compound to turn yellow.

<sup>3</sup> The Pregl determinations for carbon and hydrogen were kindly performed by Dr. Goldberg in the laboratory of Dr. M. Bergmann.

On the basis of these figures this preparation is either a 9-carbon diphosphate compound or some multiple of this, or it is a 1:1 mixture of a 6-carbon monophosphate and a 3-carbon monophosphate that crystallize together. The possibility of its being a mixture of 2 molecules of a 6-carbon monophosphate and 1 molecule of the usual 6-carbon diphosphate is ruled out by the fact that all the phosphorus is difficult to hydrolyze. No 9-carbon diphosphate compound has ever been described, but the formation of such a compound in the presence of hexosemonophosphate and triosemonophosphate seems worthy of consideration.

It seemed probable that the rate of diffusion through a glass membrane (10) might throw some light on the size of the molecule with which we were dealing. It is realized that with substances containing so many ionizable groups the rate of diffusion may be a complicated matter, so these data are not stressed here but presented for whatever they are worth. If 0.02 M potassium glycerophosphate in 1.0 M KCl is used for reference and 0.02 M potassium hexosediphosphate in 1.0 M KCl is compared with it, then, on the assumption that their rates of diffusion are inversely proportional to the square root of their molecular weights, a molecular weight of 513 is obtained for the diphosphate. The calculated value (potassium salt) is 492. In the same way if a solution of the potassium salt of our crystals 0.02 M with respect to phosphorus and 1.0 M with KCl is used, a value of 334 is obtained for the molecular weight. The calculated value for a 9-carbon compound is 584. It seems safe to conclude from this that we are not dealing with a compound that contains more than 9 carbon atoms and probably the average molecular weight is considerably less than corresponds to a 9-carbon compound.

The fact that the phosphorus is difficult to hydrolyze with acid, together with the fact that there is no alkali-labile phosphorus, rules out the possibility of glyceraldehyde phosphate or dihydroxyacetone phosphate being present. The curve obtained by titrating the free acid (prepared over the lead salt) with base shows that 2 acid equivalents per phosphorus atom are present (Fig. 2). One of these constants is that of a strong acid and the other is that of a phosphate ester; hence each phosphoric group is esterified only once and no carboxyl group is present. Consequently, of the known 3-carbon esters only glycerophosphate can be present.

The problem then is to show that these crystals are made up of a 1:1 mixture of glucosemonophosphate and glyceromonophosphate.

The separation of this preparation into fractions could not be accomplished by fractional precipitation of the calcium salt with alcohol. The analysis of each fraction is the same. Robison and King (1) obtained their pure glucosephosphate by fractionally recrystallizing the brucine salts, so it was attempted to fractionate this preparation in that way. The free acid was prepared via the lead salt and made just alkaline to litmus with brucine. After being recrystallized twice from 80 per cent ethyl alcohol, twice from 95 per cent ethyl alcohol, and twice from absolute methyl alcohol it was reconverted to the calcium salt, recrystallized from water, and dried. This had a phosphorus content of 9.58, an aldose value of 96.3 per cent, a Hagedorn-Jensen value of 80.8 per cent, and an  $[\alpha]_{546}^{\text{free acid}}$  of 40.6°. Thus it appears to be almost pure glucosemonophosphate. The aldose determinations reported here were carried out according to Macleod and Robison (11). The equivalent of 2 mg. of glucose was used, and 0.4 cc. of a 1:1 mixture of 0.5 M  $\text{NaHCO}_3$  and 0.5 M  $\text{NaCO}_3$  and 5.0 cc. of 0.02 N iodine were added. After the mixture had stood for 45 minutes at 30°, the excess iodine was titrated with 0.02 N  $\text{Na}_2\text{S}_2\text{O}_3$ . Blanks and determinations on pure glucose were run with each set. The rotation was determined by dissolving 34.57 mg. in 0.5 cc. of 1.0 N HCl. The rotation in a 0.5 dm. tube was 1.09°.

It is very difficult to obtain the non-aldose component in pure form by this brucine fractionation. The product isolated still titrates as 20.0 per cent aldose. Better success was achieved by isolating the product remaining after fermenting the aldose.

The curves shown in Fig. 1 indicate that although the fermentation of the ester starts immediately, in contrast to glucose, it practically ceases long before the  $\text{CO}_2$  production reaches the equivalent of 2 moles of  $\text{CO}_2$  per atom of added phosphorus. It is usual, even with glucose, that the fermentation is less than 100 per cent, but this fermentation is considerably less than with glucose. The remainder of the added substance remains as a compound in which the phosphorus is difficult to hydrolyze. This remaining ester was isolated from a regular preparation in which the fermentation was allowed to proceed almost to completion. It had only a slight reducing action, a carbon to phosphorus ratio

of 3.0, contained no carboxyl group, and was very difficultly hydrolyzable. It was apparently glycerophosphate. Thus the two expected components can be obtained and at least one of them, the aldose, by a method that would not be expected to split a 9-carbon compound. This fact, together with the molecular weight

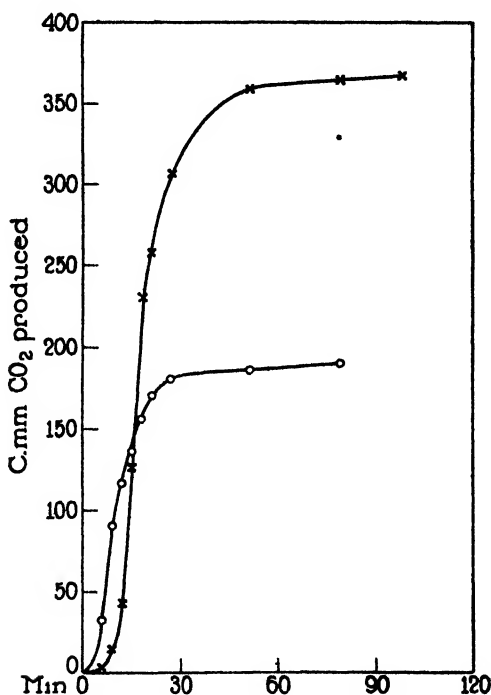


FIG. 1. The fermentation of the ester compared with glucose. The crosses indicate 0.5 cc. of Lebedev extract + 0.5 cc. of  $H_2O$  + 0.1 cc. of 0.1 M glucose; the circles 0.5 cc. of Lebedev extract + 0.5 cc. of  $H_2O$  + 0.1 cc. of potassium salt of ester 0.1 M with respect to phosphorus. The experiment was carried out with the usual Warburg apparatus. Temperature,  $28^\circ$ . These extracts produced no  $CO_2$  before the addition of a substrate.

obtained, and the fact that if a solution of the crystals is hydrolyzed with an intestinal phosphatase preparation glucosazone can be obtained from the hydrolysate,<sup>4</sup> make it appear that the original

<sup>4</sup> This experiment was kindly carried out by Dr. P. A. Levene.

preparation is a mixture of the above two components. It remains to be shown that these two substances do tend to crystallize together in a 1:1 ratio.

Preparations of hexosemonophosphate (carbon to phosphorus ratio of 6.0) and of synthetic glycerophosphate, both as calcium salts, were mixed in an equimolar ratio. Alcohol was added at room temperature to a faint turbidity and the mixture then heated on a water bath. The crystalline precipitate was filtered off and the cooled filtrate again treated with alcohol to turbidity and heated. A second fraction of crystals was then obtained. After thorough drying over  $P_2O_5$ , phosphorus was determined on each of the two precipitates. They were identical, each containing 11.28 per cent. The experiment was then repeated with 2 moles of hexosemonophosphate to 1 of glycerophosphate. The phosphorus in Fraction I was 11.25 per cent and in Fraction II 10.40 per cent. The experiment was again repeated, this time with 2 moles of glycerophosphate to 1 of hexosemonophosphate. The phosphorus in Fraction I was 11.67 per cent and in Fraction II 12.22 per cent. It thus appears that there is a strong tendency to form mixed crystals in the ratio 1:1; in other words, that mixed crystals of this ratio are more difficultly soluble than the hexosemonophosphate alone and also more insoluble than the glycerophosphate alone, although the separation is less sharp in the latter case. This fact, together with the above data, is, I believe, strong evidence that our original crystals are a 1:1 mixture of glucosemonophosphate and glycerophosphate.

As additional evidence I have determined glycerol. Meyerhof and Kiessling (12) found that glycerophosphate reacts by the Zeisel and Fanto (13) method just as does free glycerol, whereas the other phosphate esters tested do not give a positive result. I have applied this method with the Pregl (14) microapparatus and have found that our preparation always gives a positive result. Quantitatively, the results vary by several per cent, but compared with an equivalent amount of free glycerol under the same conditions, the preparation contains 18 to 19 per cent glycerol. The theoretical value is 15.5 per cent.

Although the above evidence seems ample to justify the conclusion stated, I must remark on how difficult this mixture is to separate, as some of the following experiments show.

If the preparation is heated in an acid solution, very little inorganic phosphorus is formed, as stated above, but the solution readily turns brown and if the heating is continued a brown precipitate forms. After heating for  $2\frac{1}{2}$  hours at  $100^\circ$  in 1.0 N HCl, the color was removed and the remaining esters isolated as the calcium salt. They readily crystallized, had an aldose value of 49 per cent, and a carbon to phosphorus ratio of 4.7.

After oxidation with iodine, as in the regular aldose determination, and extraction of the excess iodine with chloroform a crystalline calcium salt can be isolated from an alkaline solution. After recrystallization this contains C 17.6, P 9.9, and Ca 16.21 per cent; calculated for  $(C_9H_{22}O_{10}P_2Ca_2)_2Ca$  (a 9-carbon compound), C 17.6, P 10.12, and Ca 16.30 per cent. This substance was converted to the free acid (over the lead salt), the phosphorus determined, and then titrated with base. It was found that the phosphorus present accounted for 82.4 per cent of the total base used. The theoretical, if one carboxyl group is present per two phosphate groups, is 80.0 per cent. The presence of this carboxyl can be seen in the middle portion of the lower curve in Fig. 2.

If an aqueous solution of the calcium salt is treated with hydroxylamine hydrochloride +  $Na_2CO_3$ , allowed to stand overnight at room temperature, and then precipitated with alcohol, well formed crystals are obtained containing 2.3 per cent N. This is the theoretical figure for 1 molecule of hydroxylamine per 9 carbon atoms. However, this compound appears to be unstable. On recrystallization nitrogen is lost. This is not, however, an enrichment of the crystals with non-nitrogenous glycerophosphate, for the phosphorus does not change proportionately. Furthermore, no correspondingly richer nitrogen fraction could be obtained. As an example, a sample prepared at  $0^\circ$ , contained 2.25 per cent N. After recrystallization the N had dropped to 1.64 per cent. This preparation was again treated with hydroxylamine, let stand overnight, and crystallized by the addition of alcohol. The N not only had not returned to the original value, but had fallen further to 1.29 per cent. The phosphorus, however, was still 9.8 per cent.

If the aqueous solution of calcium salt is treated with semicarbaside, allowed to stand at room temperature for 24 hours, and then precipitated with alcohol, the precipitate contains 6.43 per



cent N. The theoretical for 1 molecule of semicarbazide per 9 carbon atoms is 6.61 per cent. After reprecipitation the N was 6.30 per cent. We were unable to crystallize this substance. It is very hygroscopic. Most of the salts prepared are stable in air when they contain  $2\text{H}_2\text{O}$  per calcium atom, but this one is not.

If an aqueous solution of the calcium salt is treated with phenylhydrazine in an acetate buffer at  $100^\circ$ , a non-crystalline precipitate

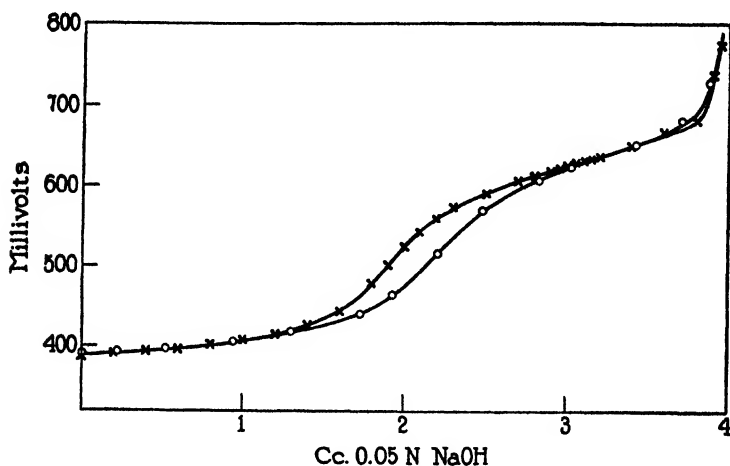


FIG. 2. The titration of the free acid of the ester with alkali. The crosses indicate the original ester; the circles the crystals isolated after oxidation with iodine. The latter curve (the circles) is made up of three parts. The first two-fifths is the strong phosphoric group, the middle one-fifth is a carboxyl group, and the last two-fifths the second phosphoric group. The experiment was arranged so that the total alkali used was the same. The potentials were measured with a hydrogen electrode and are expressed against the saturated calomel electrode.

forms. After being washed with hot alcohol and dried, this contained 10.12 per cent N and 6.82 per cent P. This is a nitrogen to phosphorus ratio of 3.24. No inorganic phosphorus is formed during the treatment and no glycerophosphate could be isolated from the filtrate. If the calcium is removed before treatment with the phenylhydrazine, a crystalline precipitate is formed. This contains 10.68 per cent N. Since the phenylhydrazine salt of glycerophosphate is not insoluble when alone, one might expect

that it would remain in solution if a mixture of it and glucose-phosphate is treated in this way. We were, however, unable to isolate any glycerophosphate from the filtrate.

The methyl ester of this preparation was prepared by treating the free acid dissolved in methyl alcohol with diazomethane in ether (15) until the solution when added to water gave a neutral reaction. The product was an oil which I was unable to crystallize. On treating this oil with acetic anhydride and pyridine, another oil was obtained which I was also unable to crystallize.

#### DISCUSSION

It seems worth pointing out that according to the method by which the preparation described here was made (it is the standard method of preparing hexosemonophosphate from yeast) we must expect a concentration of glycerophosphate to be built up. It is well known that under such conditions the concentration of hexosediphosphate is built up. According to Meyerhof and Lohmann (16) in such extracts hexosediphosphate very rapidly establishes an equilibrium with 1 molecule each of dihydroxyacetone phosphate and glyceraldehyde phosphate. Thus we must expect that if the concentration of diphosphate is high, the concentration of the triosephosphates also tends to be high. These triosephosphates can either be oxidized by the acetaldehyde to phosphoglyceric acid or 2 molecules can dismute, giving 1 of phosphoglyceric acid and 1 of glycerophosphate. This dismutation is believed to occur very readily and, if a concentration of triosephosphates is maintained, would be expected to occur continuously. The glycerophosphate is, according to Meyerhof and Kiessling (17), inert and either remains as such or is dephosphorylated. It would seem inevitable then that under conditions which permit diphosphate to accumulate, glycerophosphate must also accumulate.

#### SUMMARY

The preparation from yeast extract of a crystalline calcium salt containing 9 carbon atoms and 2 atoms of phosphorus is described. Some properties of the preparation are discussed and it is concluded that the salt is an equimolar mixture of glucosemonophosphate and glycerophosphate.

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# STUDIES ON THE MERCAPTURIC ACID SYNTHESIS IN ANIMALS

## I. THE EXTENT OF THE SYNTHESIS OF *p*-BROMOPHENYLMERCAPTURIC ACID IN DOGS AS AFFECTED BY DIETS OF VARYING SULFUR CONTENT

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One method of studying the extent of the synthesis of mercapturic acids in animals is the isolation of the acids from the urine of animals which were fed halogenated benzenes under various dietary conditions. As a quantitative procedure, such a method is unreliable. The isolation methods of Baumann and Schmitz (1) and of Baumann and Preusse (2), which are most commonly used, occasionally fail even as qualitative procedures (3). The unreliability of the isolation procedures may, perhaps, account for the conflicting results obtained by several workers (3-5).

The extent of the synthesis of mercapturic acids in animals has also been studied by the use of the so called sulfur partition method. The method is based on the assumption that the rise in the output of neutral sulfur in the urine of animals after the administration of halogenated benzenes is due to the excreted mercapturic acid only and that no effects leading to the excretion of compounds other than the mercapturic acid in the neutral sulfur fraction of the urine are produced by the administration of the halogenated benzenes. As has been pointed out in an earlier paper (6), these assumptions have no experimental justification. This method of studying the extent of the synthesis of mercapturic acids in animals is particularly troublesome when it is desired to ascertain the quantitative effects produced by *l*-cystine, *dl*-methionine, cysteine, and taurine on the extent of the synthesis of mercapturic acids. Although *l*-cystine, *dl*-methionine, and cysteine are more or less completely oxidized in the animal body

to yield inorganic sulfates in the urine, when fed in small single doses, a certain amount of evidence exists which seems to show that these amino acids are subject to incomplete oxidation and excretion in the neutral sulfur fraction of the urine (7-10). Neither is the neutral sulfur fraction of the urine entirely endogenous in origin, and it can be considerably increased by the variation in the amount and kind of protein in the diet (8, 9). With the sulfur partition method in studying the extent of the synthesis of mercapturic acids in animals under various dietary conditions, the probable interference of the factors mentioned above with the results obtained has either been considered as insignificant (10), acknowledged (11), or taken care of by the control experiments and the interpolation of the data (8), a procedure which is not entirely satisfactory, although the best possible one under the circumstances.

The recently developed method for the estimation of *p*-bromophenylmercapturic acid in dog urine (6) made it possible to check the validity of the assumptions made in the sulfur partition and the isolation methods in studying the extent of the synthesis of mercapturic acids in dogs. Using various dietary conditions, we have carried out experiments in which the rise in the output of neutral sulfur and the excretion of *p*-bromophenylmercapturic acid in the urine after the administration of bromobenzene to dogs have been compared. We have also investigated the dependence of the extent of the synthesis of *p*-bromophenylmercapturic acid in dogs on the nature of dietary protein. Similar experiments have been carried out by White and Lewis (10), who used in their study the sulfur partition method. For reasons stated above, it seemed, however, necessary to repeat the work of White and Lewis (10); the direct method for the determination of mercapturic acid in dog urine was used.

#### EXPERIMENTAL

The urine was collected every 24 hours by catheterization, at 9 a.m., from four adult female dogs which were kept in individual metabolism cages and fed the diets listed in Table I. All diets were fed in amounts yielding 60 to 65 calories per kilo of body weight once a day immediately after the catheterization. Water was allowed *ad libitum*. The food was consumed readily and at no time had we to resort to forced feeding.

The plan of the experiments was to feed the dogs the chosen diet and collect the urine every 24 hours until the output of various urinary constituents showed little or no variation from day to day; to feed 1.0 gm. of bromobenzene in a gelatin capsule at 11 a.m. to the dogs and collect and analyze the urine until the output of the same urinary constituents had returned to normal. The same plan was followed with each diet listed in Table I. The supplements of *l*-cystine, cysteine, *dl*-methionine, and taurine were weighed out and mixed with the daily portion of food.

TABLE I\*  
*Composition of Diets*

| Diet                   | Casein   | Lactalbumin | Low protein | Protein-free |
|------------------------|----------|-------------|-------------|--------------|
|                        | gm.      | gm.         | gm.         | gm.          |
| Casein†.....           | 43.7     |             | 13.0        |              |
| Lactalbumin†.....      |          | 30.0        |             |              |
| Sucrose.....           | 40.6     | 54.3        | 71.3        | 84.3         |
| Squibb's vitavose..... | 11.6     | 11.6        | 11.6        | 11.6         |
| Salt mixture‡.....     | 1.4      | 1.4         | 1.4         | 1.4          |
| Bone ash.....          | 2.7      | 2.7         | 2.7         | 2.7          |
| Butter fat.....        | 7.0      | 7.0         | 7.0         | 7.0          |
| Lard.....              | 17.0     | 17.0        | 17.0        | 17.0         |
|                        | per cent | per cent    | per cent    | per cent     |
| Nitrogen.....          | 4.84     | 3.83        | 1.46        | 0.16         |
| Sulfur.....            | 0.25     | 0.39        | 0.075       | 0.024        |

\* The diets were devised according to Cowgill, G. R., Deuel, H. J., Jr., and Smith, A. H., *Am. J. Physiol.*, **73**, 106 (1925).

† Casein No. 453 was purchased from the Casein Company of America, Inc.

‡ Lactalbumin No. 7-HAA was purchased from the National Milk Sugar Company, Inc.

§ As used by Karr, W. G., *J. Biol. Chem.*, **44**, 255 (1920).

The same methods of analysis of urine were used as those employed previously (12). *p*-Bromophenylmercapturic acid was estimated by the recently developed method (6) in which the iodine and the HgCl<sub>2</sub> procedures are employed for the same sample of urine. The mercapturic acid was determined in the urine on the day of the administration of bromobenzene and on the subsequent days until the results obtained indicated the absence of mercapturic acid in the urine. The excretion of *p*-bromophenyl-

mercapturic acid in the urine after a single feeding of 1.0 gm. of bromobenzene generally extended over 2 to 3 days. In all cases, the 24 hour sample of urine was filtered and made up to 500 cc. with distilled water.

Whenever it seemed necessary, *p*-bromophenylmercapturic acid was isolated by the method of McGuinn and Sherwin (3).

*L*-Cystine was prepared from human hair with the necessary precautions against racemization. Taurine was prepared by the method of Schmidt (13). Cysteine hydrochloride and *dl*-methionine were purchased from the Eastman Kodak Company. The purity of all compounds was checked by analysis.

### Results

For the sake of economy of space, results of the experiments on only two dogs are shown in Tables II to IV. Similar data were obtained with the other animals.

*Neutral Sulfur and Mercapturic Acid Excretion*—The data shown in Table II apparently indicate that the neutral sulfur fraction of the urine can be considerably increased by the substitution of lactalbumin for casein in the diet. Supplementing the casein diet with *L*-cystine or cysteine, in amounts yielding 267 mg. of sulfur, also raised markedly the output of neutral sulfur in the urine. The results shown in Table III indicate that *L*-cystine, cysteine, or *dl*-methionine, when administered repeatedly to a dog maintained on a low protein diet, gradually increases the output of the neutral sulfur in the urine. Taurine, when fed, was excreted almost quantitatively in the neutral sulfur fraction. On a protein-free diet (Table IV), repeated administration of the sulfur-containing amino acids did not affect appreciably the excretion of the neutral sulfur.

The output of *p*-bromophenylmercapturic acid in the urine of dogs fed the casein diet or lactalbumin diet was almost equivalent to the rise in the output of neutral sulfur. Higher values of the neutral sulfur obtained after the feeding of bromobenzene to dogs kept on the casein diet supplemented by *L*-cystine or cysteine do not indicate the increased synthesis of the mercapturic acid from the excess cystine or cysteine present in the diet (Table II). The data shown in Table IV also indicate that on a protein-free diet the rise in the output of neutral sulfur in the urine of dogs receiv-

TABLE II

*Metabolism of Bromobenzene in Dog on Casein and Lactalbumin Diets*  
Dog 14; weight 9.7 kilos.

| Day | Total N | Total S | Total SO <sub>4</sub> -S | Inorganic SO <sub>4</sub> -S | Ethereal SO <sub>4</sub> -S | Neutral S | Mercap-<br>turic<br>acid S |                   | Day | Total N | Total S | Total SO <sub>4</sub> -S | Inorganic SO <sub>4</sub> -S | Ethereal SO <sub>4</sub> -S | Neutral S | Mercap-<br>turic<br>acid S |                   |
|-----|---------|---------|--------------------------|------------------------------|-----------------------------|-----------|----------------------------|-------------------|-----|---------|---------|--------------------------|------------------------------|-----------------------------|-----------|----------------------------|-------------------|
|     |         |         |                          |                              |                             |           | I <sub>2</sub>             | HgCl <sub>2</sub> |     |         |         |                          |                              |                             |           | I <sub>2</sub>             | HgCl <sub>2</sub> |

Casein diet. Intake, 4.8 gm. N, 0.240 gm. S

|     | gm.  | mg. | mg. | mg. | mg. | mg. | mg. | mg. |     | gm.* | mg. | mg. | mg. | mg. | mg. | mg. | mg. |
|-----|------|-----|-----|-----|-----|-----|-----|-----|-----|------|-----|-----|-----|-----|-----|-----|-----|
| 14  | 4.22 | 200 | 140 | 117 | 23  | 60  |     |     | 27* | 4.28 | 234 | 105 | 59  | 46  | 129 | 70  | 72  |
| 15  | 4.46 | 195 | 129 | 105 | 24  | 66  |     |     | 28  | 4.30 | 115 | 41  | 18  | 23  | 74  | 12  | 14  |
| 16† | 4.56 | 414 | 338 | 315 | 23  | 76  |     |     | 29  | 4.40 | 177 | 113 | 91  | 22  | 64  |     |     |
| 17† | 4.36 | 448 | 343 | 321 | 22  | 105 |     |     | 30  | 4.23 | 161 | 104 | 83  | 21  | 57  |     |     |
| 18† | 4.26 | 437 | 345 | 321 | 24  | 92  |     |     | 31† | 4.63 | 393 | 311 | 287 | 24  | 82  |     |     |
| 19§ | 4.40 | 486 | 322 | 273 | 49  | 164 | 73  | 74  | 32† | 4.46 | 433 | 334 | 309 | 25  | 99  |     |     |
| 20† | 4.23 | 385 | 274 | 250 | 24  | 111 | 18  | 19  | 33† | 4.50 | 438 | 340 | 315 | 25  | 98  |     |     |
| 21† | 4.27 | 418 | 328 | 304 | 24  | 90  |     |     | 34  | 4.41 | 484 | 324 | 272 | 52  | 160 | 72  | 73  |
| 22  | 4.26 | 200 | 134 | 114 | 20  | 66  |     |     | 35† | 4.33 | 343 | 248 | 219 | 29  | 95  | 16  | 14  |
| 23  | 4.22 | 196 | 133 | 111 | 22  | 63  |     |     | 36† | 4.20 | 440 | 350 | 326 | 24  | 90  |     |     |
| 24  | 4.26 | 190 | 128 | 105 | 23  | 62  |     |     | 37  | 4.14 | 218 | 145 | 122 | 23  | 73  |     |     |
| 25  | 4.24 | 197 | 133 | 109 | 24  | 64  |     |     | 38  | 4.16 | 200 | 138 | 116 | 22  | 62  |     |     |
| 26  | 4.27 | 184 | 122 | 98  | 24  | 62  |     |     | 39  | 4.20 | 196 | 136 | 115 | 21  | 60  |     |     |

Lactalbumin diet. Intake, 3.80 gm. N, 0.390 gm. S

|     | gm.  | mg. | mg. | mg. | mg. | mg. | mg. | mg. |     | gm.  | mg. | mg. | mg. | mg. | mg. | mg. | mg. |
|-----|------|-----|-----|-----|-----|-----|-----|-----|-----|------|-----|-----|-----|-----|-----|-----|-----|
| 14  | 3.26 | 300 | 200 | 177 | 33  | 100 |     |     | 21  | 3.08 | 291 | 188 | 142 | 26  | 103 |     |     |
| 15  | 3.10 | 299 | 196 | 166 | 30  | 103 |     |     | 22  | 3.00 | 294 | 186 | 141 | 25  | 108 |     |     |
| 16  | 3.08 | 295 | 186 | 149 | 37  | 109 |     |     | 23* | 3.03 | 329 | 148 | 98  | 50  | 181 | 71  | 72  |
| 17* | 3.09 | 346 | 164 | 98  | 66  | 182 | 64  | 67  | 24  | 3.23 | 267 | 136 | 106 | 30  | 131 | 23  | 22  |
| 18  | 3.02 | 235 | 112 | 90  | 20  | 123 | 29  | 29  | 25  | 3.29 | 295 | 184 | 163 | 21  | 111 |     |     |
| 19  | 3.00 | 282 | 168 | 148 | 20  | 114 |     |     | 26  | 3.10 | 290 | 187 | 165 | 22  | 103 |     |     |
| 20  | 3.04 | 276 | 175 | 129 | 26  | 101 |     |     | 27  | 3.11 | 297 | 196 | 169 | 27  | 101 |     |     |

\* 1.0 gm. of bromobenzene in a gelatin capsule was fed at 11 a.m.

† 1.0 gm. of *l*-cystine was fed with the food; 0.267 gm. of S.

‡ 1.54 gm. of cysteine hydrochloride + 0.53 gm. of Na<sub>2</sub>CO<sub>3</sub> were fed with the food; 0.267 gm. of S.

§ 1.0 gm. of *l*-cystine was fed with the food; 1.0 gm. of bromobenzene in a gelatin capsule was fed at 11 a.m.

|| 1.54 gm. of cysteine hydrochloride + 0.53 gm. of Na<sub>2</sub>CO<sub>3</sub> were fed with the food; 1.0 gm. of bromobenzene in a gelatin capsule was fed at 11 a.m.



TABLE III\*

*Metabolism of Bromobenzene in Dog on a Low Protein Diet*

Dog 14; the initial weight of the dog was 9.7 kilos; the final weight 9.3 kilos.

| Day  | Total N |     | Total S |     | Total SO <sub>2</sub> -S |     | Inorganic SO <sub>4</sub> -S |     | Etheral SO <sub>4</sub> -S |                   | Neutral S |     | Mercap-<br>turic<br>acid S |     | Day  | Total N |     | Total S |     | Total SO <sub>2</sub> -S |     | Inorganic SO <sub>4</sub> -S |     | Etheral SO <sub>4</sub> -S |     | Neutral S |     | Mercap-<br>turic<br>acid S |                   |
|------|---------|-----|---------|-----|--------------------------|-----|------------------------------|-----|----------------------------|-------------------|-----------|-----|----------------------------|-----|------|---------|-----|---------|-----|--------------------------|-----|------------------------------|-----|----------------------------|-----|-----------|-----|----------------------------|-------------------|
|      | gm.     | mg. | mg.     | mg. | mg.                      | mg. | mg.                          | mg. | I <sub>2</sub>             | HgCl <sub>2</sub> | gm.       | mg. | mg.                        | mg. |      | mg.     | mg. | mg.     | mg. | mg.                      | mg. | mg.                          | mg. | mg.                        | mg. | mg.       | mg. | I <sub>2</sub>             | HgCl <sub>2</sub> |
| 4    | 1.80    | 87  | 27      | 10  | 17                       | 60  |                              |     |                            |                   |           |     |                            |     | 41   | 1.50    | 104 | 41      | 26  | 15                       | 63  |                              |     |                            |     |           |     |                            |                   |
| 5    | 1.81    | 86  | 29      | 13  | 16                       | 57  |                              |     |                            |                   |           |     |                            |     | 42   | 1.40    | 100 | 40      | 25  | 15                       | 60  |                              |     |                            |     |           |     |                            |                   |
| 6†   | 1.65    | 184 | 123     | 106 | 17                       | 61  |                              |     |                            |                   |           |     |                            |     | 43‡  | 1.58    | 172 | 55      | 6   | 49                       | 117 | 54                           | 56  |                            |     |           |     |                            |                   |
| 7†   | 1.43    | 213 | 154     | 138 | 16                       | 59  |                              |     |                            |                   |           |     |                            |     | 44   | 1.75    | 101 | 26      | 7   | 19                       | 75  | 28                           | 29  |                            |     |           |     |                            |                   |
| 8§   | 1.37    | 265 | 128     | 93  | 35                       | 137 | 78                           | 79  |                            |                   |           |     |                            |     | 45   | 1.78    | 93  | 26      | 10  | 16                       | 67  |                              |     |                            |     |           |     |                            |                   |
| 9†   | 1.47    | 265 | 176     | 152 | 24                       | 89  | 25                           | 23  |                            |                   |           |     |                            |     | 46   | 1.54    | 90  | 30      | 15  | 15                       | 60  |                              |     |                            |     |           |     |                            |                   |
| 10†  | 1.51    | 300 | 199     | 185 | 14                       | 101 |                              |     |                            |                   |           |     |                            |     | 47   | 1.60    | 87  | 28      | 15  | 13                       | 59  |                              |     |                            |     |           |     |                            |                   |
| 11   | 1.34    | 124 | 45      | 31  | 14                       | 79  |                              |     |                            |                   |           |     |                            |     | 48   | 1.51    | 83  | 22      | 8   | 14                       | 61  |                              |     |                            |     |           |     |                            |                   |
| 12   | 1.57    | 99  | 29      | 14  | 15                       | 70  |                              |     |                            |                   |           |     |                            |     | 49   | 1.53    | 81  | 21      | 6   | 15                       | 60  |                              |     |                            |     |           |     |                            |                   |
| 13   | 1.61    | 84  | 21      | 6   | 15                       | 63  |                              |     |                            |                   |           |     |                            |     | 50   | 1.50    | 80  | 17      | 5   | 12                       | 63  |                              |     |                            |     |           |     |                            |                   |
| 14‡  | 1.82    | 188 | 55      | 14  | 41                       | 133 | 66                           | 66  |                            |                   |           |     |                            |     | 51   | 1.49    | 80  | 20      | 6   | 14                       | 60  |                              |     |                            |     |           |     |                            |                   |
| 15   | 2.01    | 90  | 19      | 1   | 18                       | 71  | 11                           | 11  |                            |                   |           |     |                            |     | 52‡  | 1.54    | 153 | 56      | 10  | 46                       | 97  | 36                           | 38  |                            |     |           |     |                            |                   |
| 16   | 1.90    | 90  | 22      | 6   | 16                       | 68  |                              |     |                            |                   |           |     |                            |     | 53   | 1.79    | 98  | 30      | 0   | 30                       | 68  | 19                           | 16  |                            |     |           |     |                            |                   |
| 17   | 1.76    | 87  | 29      | 14  | 15                       | 58  |                              |     |                            |                   |           |     |                            |     | 54   | 1.80    | 73  | 14      | 3   | 11                       | 59  |                              |     |                            |     |           |     |                            |                   |
| 18   | 1.70    | 237 | 26      | 10  | 16                       | 211 |                              |     |                            |                   |           |     |                            |     | 55   | 1.80    | 76  | 15      | 3   | 12                       | 61  |                              |     |                            |     |           |     |                            |                   |
| 19   | 1.54    | 298 | 23      | 8   | 15                       | 275 |                              |     |                            |                   |           |     |                            |     | 56   | 1.63    | 77  | 17      | 3   | 14                       | 60  |                              |     |                            |     |           |     |                            |                   |
| 20   | 1.54    | 318 | 28      | 12  | 16                       | 290 |                              |     |                            |                   |           |     |                            |     | 57¶  | 1.22    | 178 | 161     | 146 | 15                       | 67  |                              |     |                            |     |           |     |                            |                   |
| 21** | 1.90    | 411 | 61      | 10  | 51                       | 350 | 39                           | 39  |                            |                   |           |     |                            |     | 58¶  | 1.16    | 210 | 140     | 124 | 16                       | 70  |                              |     |                            |     |           |     |                            |                   |
| 22   | 2.11    | 377 | 32      | 5   | 27                       | 345 | 11                           | 10  |                            |                   |           |     |                            |     | 59¶  | 1.18    | 277 | 184     | 171 | 13                       | 93  |                              |     |                            |     |           |     |                            |                   |
| 23   | 2.18    | 365 | 22      | 9   | 13                       | 343 |                              |     |                            |                   |           |     |                            |     | 60†† | 1.21    | 325 | 151     | 111 | 40                       | 174 | 70                           | 72  |                            |     |           |     |                            |                   |
| 24   | 1.96    | 305 | 19      | 2   | 17                       | 286 |                              |     |                            |                   |           |     |                            |     | 61¶  | 1.20    | 260 | 127     | 110 | 17                       | 133 | 24                           | 26  |                            |     |           |     |                            |                   |
| 25   | 1.82    | 330 | 21      | 6   | 15                       | 309 |                              |     |                            |                   |           |     |                            |     | 62¶  | 1.18    | 310 | 210     | 196 | 14                       | 100 |                              |     |                            |     |           |     |                            |                   |
| 26   | 1.67    | 319 | 17      | 5   | 12                       | 302 |                              |     |                            |                   |           |     |                            |     | 63   | 1.33    | 120 | 130     | 114 | 16                       | 90  |                              |     |                            |     |           |     |                            |                   |
| 27** | 1.99    | 413 | 53      | 6   | 47                       | 360 | 37                           | 39  |                            |                   |           |     |                            |     | 64   | 1.47    | 90  | 29      | 14  | 15                       | 61  |                              |     |                            |     |           |     |                            |                   |

\* Intake, 1.46 gm. of N, 0.075 gm. of S.

† 1.0 gm. of *l*-cystine was fed with the food; 0.267 gm. of S.

‡ 1.0 gm. of bromobenzene in a gelatin capsule was fed at 11 a.m.

§ 1.0 gm. of *l*-cystine was fed with the food; 1.0 gm. of bromobenzene in a gelatin capsule fed at 11 a.m.

|| 1.04 gm. of taurine were fed with the food; 0.267 gm. of S.

¶ 1.34 gm. of *dl*-methionine were fed with the food; 0.267 gm. of S.

\*\* 1.04 gm. of taurine were fed with the food; 1.0 gm. of bromobenzene in a gelatin capsule fed at 11 a.m.

†† 1.34 gm. of *dl*-methionine were fed with the food; 1.0 gm. of bromobenzene in a gelatin capsule fed at 11 a.m.

TABLE III—*Concluded*

| Day  | Total N |     | Total S |     | Total SO <sub>4</sub> -S |     | Inorganic SO <sub>4</sub> -S |     | Ethereal SO <sub>4</sub> -S |      | Neutral S |     | Mercap-<br>turic<br>acid S |                   | Day | Total N |     | Total S |     | Total SO <sub>4</sub> -S |     | Inorganic SO <sub>4</sub> -S |     | Ethereal SO <sub>4</sub> -S |     | Neutral S |     | Mercap-<br>turic<br>acid S |                   |
|------|---------|-----|---------|-----|--------------------------|-----|------------------------------|-----|-----------------------------|------|-----------|-----|----------------------------|-------------------|-----|---------|-----|---------|-----|--------------------------|-----|------------------------------|-----|-----------------------------|-----|-----------|-----|----------------------------|-------------------|
|      | gm.     | mg. | gm.     | mg. | gm.                      | mg. | gm.                          | mg. | gm.                         | mg.  | gm.       | mg. | I <sub>2</sub>             | HgCl <sub>2</sub> |     | gm.     | mg. | gm.     | mg. | gm.                      | mg. | gm.                          | mg. | gm.                         | mg. | gm.       | mg. | I <sub>2</sub>             | HgCl <sub>2</sub> |
| 28   | 2.18    | 361 | 30      | 7   | 23                       | 331 | 18                           | 16  | 65                          | 1.53 | 80        | 18  | 4                          | 14                | 62  |         |     |         |     |                          |     |                              |     |                             |     |           |     |                            |                   |
| 29   | 2.09    | 351 | 16      | 3   | 13                       | 335 |                              |     | 66                          | 1.51 | 77        | 18  | 4                          | 14                | 59  |         |     |         |     |                          |     |                              |     |                             |     |           |     |                            |                   |
| 30   | 1.90    | 310 | 14      | 0   | 14                       | 296 |                              |     | 67                          | 1.60 | 73        | 13  | 3                          | 10                | 60  |         |     |         |     |                          |     |                              |     |                             |     |           |     |                            |                   |
| 31   | 1.80    | 96  | 16      | 1   | 15                       | 80  |                              |     | 68                          | 1.57 | 76        | 18  | 4                          | 14                | 58  |         |     |         |     |                          |     |                              |     |                             |     |           |     |                            |                   |
| 32   | 1.79    | 92  | 18      | 5   | 13                       | 74  |                              |     | 69                          | 1.66 | 77        | 16  | 3                          | 13                | 61  |         |     |         |     |                          |     |                              |     |                             |     |           |     |                            |                   |
| 33†† | 1.53    | 187 | 121     | 107 | 14                       | 66  |                              |     | 70                          | 1.60 | 76        | 16  | 4                          | 12                | 60  |         |     |         |     |                          |     |                              |     |                             |     |           |     |                            |                   |
| 34†† | 1.50    | 307 | 195     | 180 | 15                       | 112 |                              |     | 71                          | 1.63 | 79        | 18  | 4                          | 14                | 61  |         |     |         |     |                          |     |                              |     |                             |     |           |     |                            |                   |
| 35§§ | 1.43    | 356 | 209     | 160 | 49                       | 147 | 67                           | 69  | 72†                         | 1.96 | 161       | 50  | 1                          | 49                | 111 | 37      | 39  |         |     |                          |     |                              |     |                             |     |           |     |                            |                   |
| 36†† | 1.30    | 310 | 197     | 175 | 22                       | 103 | 33                           | 34  | 73                          | 2.00 | 90        | 17  | 4                          | 13                | 73  | 12      | 13  |         |     |                          |     |                              |     |                             |     |           |     |                            |                   |
| 37†† | 1.33    | 250 | 151     | 137 | 14                       | 99  |                              |     | 74                          | 1.97 | 73        | 14  | 3                          | 11                | 59  |         |     |         |     |                          |     |                              |     |                             |     |           |     |                            |                   |
| 38†† | 1.34    | 297 | 211     | 196 | 15                       | 86  |                              |     | 75                          | 1.80 | 72        | 12  | 0                          | 12                | 60  |         |     |         |     |                          |     |                              |     |                             |     |           |     |                            |                   |
| 39   | 1.54    | 120 | 50      | 34  | 16                       | 70  |                              |     |                             |      |           |     |                            |                   |     |         |     |         |     |                          |     |                              |     |                             |     |           |     |                            |                   |
| 40   | 1.61    | 107 | 42      | 27  | 15                       | 65  |                              |     |                             |      |           |     |                            |                   |     |         |     |         |     |                          |     |                              |     |                             |     |           |     |                            |                   |

†† 1.54 gm. of cysteine hydrochloride + 0.53 gm. of Na<sub>2</sub>CO<sub>3</sub> were fed with the food; 0.267 gm. of S.

§§ 1.54 gm. of cysteine hydrochloride + 0.53 gm. of Na<sub>2</sub>CO<sub>3</sub> were fed with the food; 1.0 gm. of bromobenzene in a gelatin capsule fed at 11 a.m.

ing bromobenzene is considerably greater than can be accounted for by the excretion of mercapturic acid. On the unsupplemented low protein diet (Table III), as in the case of the unsupplemented casein or lactalbumin diets (Table II), the rise in the output of neutral sulfur produced by the administration of bromobenzene is almost exactly accounted for by the excreted mercapturic acid. Supplementing the low protein diet (Table III) or the protein-free diet (Table IV) with *l*-cystine, cysteine, or *dl*-methionine, but not with taurine, definitely increased the output of mercapturic acid after the administration of bromobenzene.

**Ethereal Sulfate Formation and Dietary Sulfur**—The excretion of ethereal sulfates after feeding bromobenzene to dogs was definitely greater on the protein-free diet than on any other diet which we have used. With the prolonged maintenance on the protein-free diet, the dogs yielded considerably greater amounts of ethereal sulfates than after the first few days of the experiment (compare the 5th with the 24th day, Table IV).

*Total Nitrogen and Total Sulfur Excretion and Dietary Sulfur*—On the casein and lactalbumin diets or on the low protein and protein-free diets supplemented with *l*-cystine, cysteine, or meth-

TABLE IV\*

*Metabolism of Bromobenzene in Dog on Protein-Free Diet*

Dog 21; the initial weight of the dog was 8.3 kilos; the final weight, 7.8 kilos.

| Day | Total N |     | Total S |     | Total SO <sub>4</sub> -S |     | Inorganic SO <sub>4</sub> -S |     | Etheral SO <sub>4</sub> -S |     | Neutral S |     | Mercap-<br>turic<br>acid S |                   | Day | Total N |     | Total S |     | Total SO <sub>4</sub> -S |     | Inorganic SO <sub>4</sub> -S |     | Etheral SO <sub>4</sub> -S |     | Neutral S |     | Mercap-<br>turic<br>acid S |     |
|-----|---------|-----|---------|-----|--------------------------|-----|------------------------------|-----|----------------------------|-----|-----------|-----|----------------------------|-------------------|-----|---------|-----|---------|-----|--------------------------|-----|------------------------------|-----|----------------------------|-----|-----------|-----|----------------------------|-----|
|     | gm.     | mg. | gm.     | mg. | gm.                      | mg. | gm.                          | mg. | gm.                        | mg. | gm.       | mg. | I <sub>2</sub>             | HgCl <sub>2</sub> |     | gm.     | mg. | gm.     | mg. | gm.                      | mg. | gm.                          | mg. | gm.                        | mg. | gm.       | mg. | gm.                        | mg. |
| 3   | 1.16    | 96  | 33      | 14  | 19                       | 63  |                              |     |                            |     |           |     |                            |                   | 19  | 1.37    | 95  | 31      | 9   | 22                       | 64  |                              |     |                            |     |           |     |                            |     |
| 4   | 1.10    | 97  | 37      | 19  | 18                       | 60  |                              |     |                            |     |           |     |                            |                   | 20  | 1.41    | 77  | 19      | 2   | 17                       | 58  |                              |     |                            |     |           |     |                            |     |
| 5†  | 1.63    | 185 | 65      | 6   | 59                       | 120 | 38                           | 40  |                            |     |           |     |                            |                   | 21  | 1.26    | 95  | 39      | 20  | 19                       | 56  |                              |     |                            |     |           |     |                            |     |
| 6   | 1.48    | 110 | 23      | 0   | 23                       | 87  | 17                           | 15  |                            |     |           |     |                            |                   | 22  | 1.17    | 110 | 54      | 33  | 21                       | 56  |                              |     |                            |     |           |     |                            |     |
| 7   | 1.40    | 86  | 17      | 0   | 17                       | 69  |                              |     |                            |     |           |     |                            |                   | 23  | 0.97    | 84  | 28      | 10  | 18                       | 56  |                              |     |                            |     |           |     |                            |     |
| 8   | 1.37    | 84  | 23      | 4   | 19                       | 61  |                              |     |                            |     |           |     |                            |                   | 24† | 1.46    | 180 | 84      | 11  | 73                       | 96  | 21                           | 21  |                            |     |           |     |                            |     |
| 9‡  | 1.00    | 250 | 184     | 162 | 22                       | 64  |                              |     |                            |     |           |     |                            |                   | 25  | 1.57    | 124 | 41      | 1   | 40                       | 83  | 16                           | 17  |                            |     |           |     |                            |     |
| 10‡ | 0.96    | 260 | 190     | 171 | 19                       | 70  |                              |     |                            |     |           |     |                            |                   | 26  | 1.71    | 116 | 34      | 10  | 24                       | 82  |                              |     |                            |     |           |     |                            |     |
| 11§ | 1.00    | 276 | 144     | 92  | 52                       | 132 | 46                           | 43  |                            |     |           |     |                            |                   | 27  | 1.41    | 87  | 27      | 6   | 21                       | 60  |                              |     |                            |     |           |     |                            |     |
| 12‡ | 0.98    | 252 | 147     | 137 | 10                       | 105 | 32                           | 31  |                            |     |           |     |                            |                   | 28  | 0.94    | 169 | 100     | 77  | 23                       | 69  |                              |     |                            |     |           |     |                            |     |
| 13‡ | 0.96    | 211 | 152     | 129 | 23                       | 59  |                              |     |                            |     |           |     |                            |                   | 29  | 0.83    | 220 | 160     | 140 | 20                       | 60  |                              |     |                            |     |           |     |                            |     |
| 14‡ | 0.99    | 242 | 176     | 160 | 16                       | 66  |                              |     |                            |     |           |     |                            |                   | 30  | 0.83    | 224 | 166     | 145 | 21                       | 58  |                              |     |                            |     |           |     |                            |     |
| 15  | 1.03    | 193 | 130     | 112 | 18                       | 63  |                              |     |                            |     |           |     |                            |                   | 31¶ | 0.97    | 248 | 110     | 60  | 50                       | 138 | 60                           | 61  |                            |     |           |     |                            |     |
| 16  | 1.00    | 141 | 81      | 64  | 17                       | 60  |                              |     |                            |     |           |     |                            |                   | 32  | 0.93    | 200 | 110     | 83  | 27                       | 90  | 16                           | 16  |                            |     |           |     |                            |     |
| 17† | 1.57    | 194 | 75      | 17  | 58                       | 119 | 33                           | 34  |                            |     |           |     |                            |                   | 33  | 0.90    | 267 | 197     | 177 | 20                       | 70  |                              |     |                            |     |           |     |                            |     |
| 18  | 1.70    | 111 | 25      | 0   | 25                       | 86  | 18                           | 18  |                            |     |           |     |                            |                   | 34  | 1.00    | 100 | 39      | 20  | 19                       | 61  |                              |     |                            |     |           |     |                            |     |

\* Intake, 0.16 gm. of N, 0.024 gm. of S.

† 1.0 gm. of bromobenzene in a gelatin capsule was fed at 11 a.m.

‡ 1.13 gm. of *dl*-methionine were fed with the food; 0.225 gm. of S.

§ 1.13 gm. of *dl*-methionine were fed with the food; 1.0 gm. of bromobenzene fed at 11 a.m.

|| 1.0 gm. of *l*-cystine was fed with the food; 0.267 gm. of S.

¶ 1.0 gm. of *l*-cystine was fed with the food; 1.0 gm. of bromobenzene fed at 11 a.m.

ionine, but not with taurine, feeding of bromobenzene in 1.0 gm. doses did not affect the excretion of total nitrogen in the urine. In all cases, however, the excretion of total sulfur was raised on the day of administration of bromobenzene. This rise in the output of

total sulfur in the urine was compensated in all cases by a corresponding retention of sulfur on the following day. On the low protein (Table III) or the protein-free diet (Table IV), without the supplements, administration of bromobenzene produced a marked rise in the output of total nitrogen (accounted for as urea) and total sulfur in the urine. This rise in total nitrogen and sulfur was not compensated by a corresponding retention of either nitrogen or sulfur on the following day.

*Maintenance on Low Protein and Protein-Free Diets and Mercapturic Acid Excretion*—The data in Table III show that when bromobenzene was fed to dogs 4 to 5 days after the supplements of *L*-cystine (14th day of experiment) or cysteine (43rd day) have been withdrawn from the diet, considerably greater amounts of *p*-bromophenylmercapturic acid were excreted as compared to the amounts excreted after the feeding of the same dose of bromobenzene on the 10th (72nd day of experiment) or the 15th (52nd day of experiment) day after the removal of the supplements from the diet. The same relationship was observed in the case of the protein-free diet (Table IV; compare the 17th and the 24th days). It is probable that the decrease in the extent of the synthesis of the mercapturic acid with the maintenance of the animal on an inadequate diet is due to the *gradual depletion* of the sulfur stores of the animal which are used for the detoxication of bromobenzene.

Inasmuch as there remained a probability that the substance which was excreted in the urine after feeding bromobenzene to dogs maintained on a protein-free diet supplemented with *DL*-methionine was not *p*-bromophenylmercapturic acid but a similar conjugation product of bromobenzene with some intermediary breakdown product of methionine, such as homocysteine (14), as suggested by White and Lewis (10), it seemed necessary to isolate the substance from the urine. The product isolated proved to be identical with *p*-bromophenylmercapturic acid.

Yield, 120 mg.

|                 | M.p. | N    | S     | Mol. wt. |
|-----------------|------|------|-------|----------|
|                 | °C.  |      |       |          |
| Calculated..... | 152  | 4.40 | 10.06 | 318      |
| Found.....      | 152  | 4.37 | 10.00 | 314      |

## DISCUSSION

The results presented above indicate that the changes in the excretion of the neutral sulfur of the urine of dogs induced by bromobenzene feeding are not always a reliable quantitative criterion of the extent of the synthesis of *p*-bromophenylmercapturic acid. In accord with several workers (7-10), *l*-cystine and *dl*-methionine, when administered in apparently excessive doses to dogs maintained on adequate diets, increase the output of the neutral sulfur in the urine. Taurine is excreted unchanged in the neutral sulfur fraction of the urine (15).

In accord with the conclusions of other workers (8, 10), *l*-cystine and *dl*-methionine augmented the synthesis of *p*-bromophenylmercapturic acid in dogs when these amino acids were added to a low protein or a protein-free diet. Cysteine, but not taurine, was similar to *l*-cystine and *dl*-methionine in this respect. It has been shown that *l*-cystine is convertible to cysteine (16) and *vice versa* (17, 18) in the animal body. Methionine, homocysteine, and cysteine are believed to give rise to cystine in cystinurics (18). Our data may be interpreted to indicate the conversion of *l*-cystine and *dl*-methionine to cysteine. However, neither our results nor those of White and Lewis (10) demonstrate the direct union of administered bromobenzene with cysteine.

The sparing action of *l*-cystine and *dl*-methionine on the tissue nitrogen when these amino acids were fed together with bromobenzene to dogs kept on a low sulfur diet has been interpreted by White and Lewis (10) to indicate the utilization of the amino acids for the detoxication of bromobenzene in preference to the tissue sulfur. They have also suggested "that cystine and methionine may have some common product of intermediary metabolism which is essential for the normal function of the organism and that when methionine is supplied by the diet, the cystine present is thereby made available for the detoxication of the benzene derivatives." The correction of the negative nitrogen balance by *l*-cystine in adult dogs kept on low sulfur diets (19) and the increased utilization of food nitrogen by the growing dog kept on an inadequate diet, when *l*-cystine or *dl*-methionine was added to the diet (20), have been interpreted to indicate the utilization of the amino acids for replacement of tissue waste and for the synthesis of new tissue respectively. It is, perhaps, justifiable to apply a

similar interpretation to the observed sparing action of *l*-cystine, cysteine, and *dl*-methionine on the tissue nitrogen of dogs which was accompanied by a retention of the sulfur of the ingested amino acids, when bromobenzene was fed to the dogs while kept on a low protein or a protein-free diet. It is probable that under all dietary conditions which we used in the present study, bromobenzene attacked the tissue sulfur and utilized its cystine for detoxication purposes (the rise in the output of total sulfur in the urine) and the nitrogenous breakdown products of the attacked tissue were either utilized by the animal for the resynthesis of the attacked tissue in the presence of an adequate supply of organic sulfur in the diet (no effect on the urinary nitrogen and the retention of sulfur on the following day) or they were excreted in the urine as urea if the diet was too low in sulfur to effect the resynthesis of the attacked tissue (rise in the output of urea in the urine). The data in Table IV (28th day) show that *l*-cystine and *dl*-methionine arrest the breakdown of tissue (as indicated by increased nitrogen output) caused by bromobenzene feeding *after the synthesis and excretion of p-bromophenylmercapturic acid have been completed*. The probable attack on the tissue by bromobenzene, in spite of the constancy in the excretion of urinary nitrogen, is further suggested by the increased output of neutral sulfur in the urine of dogs kept on a protein-free diet supplemented by *l*-cystine and *dl*-methionine, which could not entirely be accounted for by the excretion of mercapturic acid.

#### SUMMARY

1. Bromobenzene was fed to adult dogs which were kept on diets of varying sulfur content and the excretion of neutral sulfur in the urine was compared with that of *p*-bromophenylmercapturic acid. Under certain dietary conditions, the rise in the output of neutral sulfur induced by bromobenzene feeding was found to be an unreliable criterion of the extent of the synthesis of *p*-bromophenylmercapturic acid in dogs.

2. Cysteine, *l*-cystine, and *dl*-methionine, but not taurine, augmented the synthesis of mercapturic acid in dogs maintained on low sulfur diets.

3. The results obtained seem to suggest that the augmentation of the synthesis of *p*-bromophenylmercapturic acid in dogs by

*l*-cystine, cysteine, and *dl*-methionine may possibly be a result of the replenishment of the animals' tissue sulfur which is used in the detoxication of bromobenzene, rather than evidence of the direct union of these amino acids with bromobenzene in preference to the tissue sulfur.

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# THE USE OF IODINE AND OTHER MODIFICATIONS IN THE VAN SLYKE MANOMETRIC AMINO NITROGEN METHOD

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In Van Slyke's original paper (1) on the nitrous acid method for determining amino nitrogen it was shown that cystine and glycine react peculiarly, in that they evolve, under the conditions of the determination, 6 to 8 per cent more  $N_2$  than the theoretical amount. Other naturally occurring amino acids were found to react quantitatively with their  $\alpha$ -amino groups, and with no other groups except the  $\omega$  group of lysine. Glycyl peptides (1), glutathione (2), and amino purines (3) give high results, as do cystine and glycine.

In this paper a method is described involving the use of potassium iodide in the reaction mixture, by which theoretical results are obtained with cystine and glycine, as well as with leucine, glutamic acid, and alanine. A slight modification in the construction and use of the Hempel pipette for the manometric procedure is described, which we feel makes the manipulation more reliable.

Studies were also carried out to determine the feasibility and accuracy of methods in which the entire reaction, the absorption as well as the liberation of nitric oxide, could be carried out in the reaction chamber, and the use of the Hempel pipette avoided. Van Slyke's suggestion ((4) p. 427) involving the use of the Harington-Van Slyke (5) chamber and the absorption of the nitric oxide by permanganate, added to the chamber, was found to provide a convenient, reliable, and accurate method with several slight changes. We prefer to use  $N$  NaOH to wash the chamber instead of  $5 N$ , and we use a phosphate-nitrate-permanganate saturated

with air instead of the gas-free sodium hydroxide-permanganate suggested by Van Slyke.<sup>1</sup>

The method with the Harington-Van Slyke chamber is the authors' method of choice. The Hempel pipette methods are reliable and practically as convenient, but their successful use requires a little more experience and attention to detail.

Any of the above methods may be used with or without KI and the effect of the iodide is invariably the same, to make the values for cystine<sup>2</sup> and glycine agree with the theoretical. Tryptophane is one amino acid yielding non-theoretical values, 40 to 60 per cent, when KI is used. Leucine, alanine, glutamic acid, lysine, histidine, and tyrosine yield identical values with or without KI.

The effectiveness of different amounts of KI was studied. 1 cc. of 1 per cent KI, with 5 cc. of amine in water, 1 cc. of glacial acetic acid, and 2 cc. of saturated  $\text{NaNO}_2$ , is the least amount which will give theoretical values with cystine and glycine (with cystine, 1 cc. of 0.2 per cent KI yields 104 per cent, 1 cc. of 0.02 per cent KI yields 124 per cent of the theoretical) and 1 cc. of 2 per cent KI, the

<sup>1</sup> We have also developed a method for carrying out the entire reaction in the chamber of Van Slyke and Neill without the Hempel pipette. The reaction mixture of amine and nitrous acid, after liberation of the nitric oxide and nitrogen, is made alkaline in the presence of hypobromite (the hypobromite prevents formation of  $\text{N}_2\text{O}$  and  $\text{N}_2$  by action of strong alkali on  $\text{NO}$  (6, 7)) and is then treated with an excess of oxygen, which converts the nitric oxide into nitrogen dioxide, which in turn is quantitatively absorbed by alkali. The excess oxygen is absorbed by hydrosulfite, leaving nitrogen as the residual gas.

This oxygen-hydrosulfite method yields higher than theoretical values with tyrosine (150 per cent) and tryptophane (127 per cent) owing apparently to substitution of the nitroso group in the aromatic ring and the subsequent reduction of this to nitrogen by the hydrosulfite.

Although this method is reliable with pure aliphatic amino acids, it is not recommended. Beside its inapplicability to the analysis of aromatic amino acids, it is time-consuming and the urea and ammonia corrections are large and variable, and it cannot thus be used for the direct analysis of blood filtrates.

<sup>2</sup> By Van Slyke's manometric method (without KI) we find cystine yields about 140 per cent of theoretical  $\text{N}_2$ , while Van Slyke by his original method (1) reports about 108 per cent. This discrepancy is doubtless due to the differences in the conditions of the two methods. The concentration of reagents is practically the same in both; but mercury is in contact with them in the manometric method.

amount prescribed in the procedure below, provides a definite excess. This is about the minimal amount which causes precipitation of free iodine when mixed with nitrous acid under the conditions of this analysis. Greater amounts of KI, up to 1 cc. of 40 per cent, also give theoretical values with cystine and glycine. They are, however, undesirable, for aside from causing wasteful precipitation of iodine and increasing greatly the amount of nitric oxide evolved, with certain amino acids (and urea) the rate of nitrogen formation is greatly decreased, so that with alanine, for example, 10 minutes must be allowed at 22° in order to get a theoretical nitrogen value. With 1 cc. of 2 per cent KI even alanine, the most slowly reacting amino acid in our series, gives theoretical values in the time intervals specified by Van Slyke ((4) p. 440) (5 minutes at 17.5°, 3 minutes at 25°, etc.). Urea, under these conditions, yields 5.5 per cent of its nitrogen and this is the urea correction which must be applied when blood filtrates are analyzed by the KI methods given.

In the presence of larger amounts of KI, urea liberates less nitrogen; with 1 cc. of 20 per cent KI, only 1 per cent in 3 minutes at 25° (Van Slyke's time and temperature). This is a negligible urea correction for normal blood filtrates, but this amount of KI with these time intervals may not be used with blood filtrates, since certain amino acids yield nitrogen incompletely under these conditions: alanine, 93 per cent, and leucine, 98 per cent. When with 1 cc. of 20 per cent KI the time is increased to 7.5 minutes at 25°, so that alanine and leucine give theoretical values, the urea correction is increased to 3.5 per cent. It is possible to make accurate blood filtrate amino nitrogen determinations with 1 cc. of 20 per cent KI, and we have determined in detail the times and temperatures which will give nitrogen equal to 3.5 per cent of the urea. These are 17.8 minutes at 15°, 11.5 minutes at 20°, 7.5 minutes at 25°, 4.8 minutes at 30°. We prefer, however, for blood filtrates as well as for all other amino nitrogen work with KI, to use Van Slyke's times and temperatures ((4) p. 440) with 1 cc. of 2 per cent KI in 97 per cent acetic acid, under which conditions urea yields 5.5 per cent of its nitrogen.

In studying the mechanism of action of the KI, it was found that an equimolecular amount of iodine has the same effect as iodide in giving theoretical values with cystine and glycine. Since the

iodide, when mixed with nitrous acid, is immediately oxidized to iodine, one may conclude that it is the oxidizing action of the iodine, rather than the reducing action of the iodide, which is the effective agent. This is supported by the observation that if in place of 1 cc. of 20 per cent (1.2 N) KI, 1 cc. of 0.6 N KI and 0.6 N  $\text{Na}_2\text{S}_2\text{O}_3$  is used, under which condition practically no iodine is liberated by the nitrous acid, the values with cystine are about 140 per cent, the same as by Van Slyke's method in which no iodide is used. Since the ferric-ferrous system has approximately the same oxidation-reduction potential as the iodine-iodide system, it was thought that these ions might be of value here. However, ferric or ferrous chlorides, in a wide range of concentration, from 1 cc. of 0.12 M to 1 cc. of 1.2 M, have no effect on the  $\text{N}_2$  values obtained from cystine with nitrous acid, the values being about 140 per cent, just as when no addition is made. We have also observed that when cystine is treated with nitrous acid in the presence of iodide (or iodine), sulfate is formed, just as is the case in the absence of iodide, the evidence being the white precipitate with barium chloride (8, 9).

It is generally agreed (1, 8, 9) that there are present in cystine and glycine some "abnormal" reducing groups which are not present in the other amino acids. In cystine, this abnormal group is doubtless the sulfide sulfur. In the absence of iodine this abnormal group reduces the nitrous acid with the formation of extra nitrogen. We believe that in the presence of suitable amounts of iodine, this abnormal group is oxidized before it can react appreciably with the nitrous acid, and thus theoretical values are obtained.

Comparative studies of the same blood filtrates by different methods (Table I) show that the KI methods yield consistently lower results, by 8 to 15 per cent, than do Van Slyke's method and the other non-KI methods. This agrees with the comparison made by Van Slyke and Kirk ((10) p. 675), on analysis of amino nitrogen of blood by different methods, where it is found that the values obtained by Van Slyke's manometric method are consistently higher, by 5 to 14 per cent, than the values obtained by the formol titration. This is what we should expect if blood filtrates contain significant amounts of cystine, glycine, and other compounds known to yield greater than theoretical values by Van

Slyke's amino nitrogen methods. The explanation may lie in part in such compounds as tryptophane, which show too low results by the KI methods. A similar comparison made with the use of 1 cc. of 20 per cent KI, in which the urea corrections are 3.5 per cent (instead of 1 cc. of 2 per cent KI), has shown essentially the same difference between the KI and the non-KI methods, from 8 to 14 per cent. More detailed study of the analysis of

TABLE I

*Effect of KI upon Amino Nitrogen Values of Blood Filtrates*

Method A is Van Slyke's manometric method. In Method B the Harington-Van Slyke chamber is used, and the NO is absorbed directly in the chamber with  $\text{KMnO}_4$ . Methods AA and BB are the same as Methods A and B respectively, except that KI is added.

|                    | Amino N per 100 cc. blood |          |           |           | Per cent by which Method A or B exceeds AA or BB | Urea N per 100 cc. blood |
|--------------------|---------------------------|----------|-----------|-----------|--|--------------------------|
|                    | Method A                  | Method B | Method AA | Method BB |  |                          |
|                    | mg.                       | mg.      | mg.       | mg.       |  | mg.                      |
| Human, normal..... |                           | 9.86     |           | 8.91      | 10.7   | 15.0                     |
| " " .....          |                           | 9.13     |           | 8.24      | 10.8   | 12.2                     |
| " " .....          |                           | 10.08    |           | 9.12      | 10.5   | 9.03                     |
| " uremic.....      | 8.41                      | 8.25     | 7.75      | 7.36      | 10.3   | 45.9                     |
| " " .....          |                           | 8.29     |           | 7.20      | 15.1   | 53.2                     |
| Dog 7.....         | 10.93                     | 10.68    | 9.93      | 9.49      | 11.2   | 16.8                     |
| " 9.....           | 13.49                     | 13.67    | 12.50     | 12.45     | 9.0  | 24.2                     |
| " 10.....          | 12.99                     | 12.55    | 11.70     | 11.47     | 10.2   | 16.5                     |
| " 11.....          |                           | 11.49    | 10.69     | 10.61     | 7.8  | 14.3                     |
| " 13.....          | 14.15                     | 13.79    | 12.19     | 12.04     | 15.3   | 12.0                     |

All these amino nitrogen figures are corrected for urea nitrogen, Methods A and B by 7 per cent, Methods AA and BB by 5.5 per cent.

blood filtrates by these methods will be reported in a subsequent paper.

Studies on hair hydrolysates have shown that the amino nitrogen values without KI are from 8 to 11 per cent higher, and with gelatin hydrolysates (in which about 25 per cent of the nitrogen is in glycine) from 5 to 10 per cent higher than the values with KI.

In the following pages three modified methods are described: (a) the original Van Slyke method plus KI, (b) the method with the Harington-Van Slyke chamber and KI, (c) the method with

the Harington-Van Slyke chamber without KI. Finally there is described a modified Hempel pipette.

#### *Original Van Slyke Method Plus KI*

This procedure is exactly the same as Van Slyke's manometric method (4), with the following modifications.

In place of glacial acetic acid as a reagent, a solution containing 2 per cent KI in acetic acid is used. 2 gm. of KI are dissolved in 2 cc. of water and made up to 100 cc. with glacial acetic acid.

After the nitrous acid reaction mixture has been expelled from the chamber, the chamber is washed once with 5 per cent sodium thiosulfate to remove iodine, before the usual two washings with water.

#### *Method with Harington-Van Slyke Chamber and KI*

*Reagents*—The saturated sodium nitrite and 2 per cent KI in acetic acid are identical with those described above.

Permanganate-phosphate-nitrate solution. To 5 gm. of  $\text{KMnO}_4$ , 18 gm. of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  (or 7 gm. of anhydrous  $\text{Na}_2\text{HPO}_4$ ), and 60 gm. of  $\text{NaNO}_3$  add 100 cc. of water and warm with stirring until all is in solution. This solution should be kept above  $20^\circ$ , otherwise some of the salts will crystallize out. The 5 per cent  $\text{KMnO}_4$  in 10 per cent  $\text{NaOH}$  described by Van Slyke (4) may be used here with the same accuracy, but we prefer the phosphate-nitrate-permanganate because there is less fouling of the mercury by the reagent. A phosphate-permanganate solution without nitrate may also be used, but the blanks are much higher, owing to the larger amount of dissolved gas in this reagent.

Gas-free  $\text{N NaOH}$ . 4 per cent  $\text{NaOH}$  is made gas-free by boiling or by repeatedly evacuating and expelling the extracted gases in the Van Slyke-Neill gas apparatus, after which it is stored in a gas-free reagent container over mercury.

A convenient form of gas-free reagent container, which we have been using for several years, is described in Fig. 1 (11). It is somewhat more easily made than those described by Guest and Holmes (12). It may be supported when not in use by a small conveniently portable wooden rack. The delivery capillary is so made that it fits into the bottom of the cup of the Van Slyke-Neill reaction chamber; and liquid may be caused to pass in either direction without exposure to air.

*Procedure*

The first two steps in this analysis, namely the removal of air from the mixture of amine, acetic acid, and iodide and the decomposition of the amino groups, are the same as in the above method.

*Removal of Nitrous Acid Solution and Washing of Chamber*—After the time to be allowed for the decomposition of the amino groups is complete, the leveling bulb is held in a position about 40 cm. below the 50 cc. mark. With the leveling bulb stop-cock

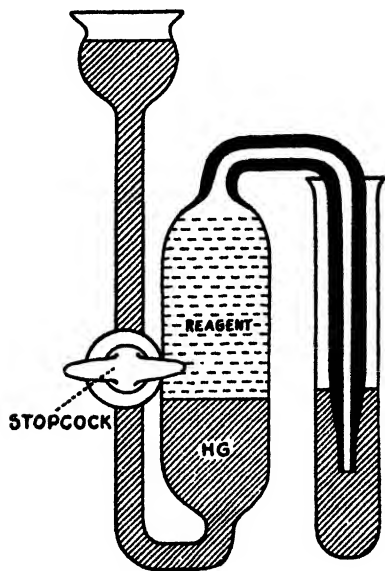


FIG. 1

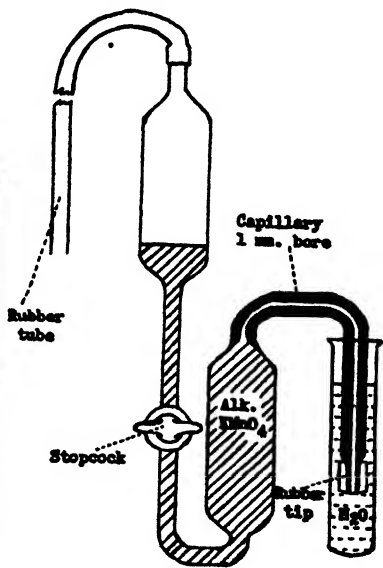


FIG. 2

FIG. 1. Pipette for storing gas-free reagents over mercury.

FIG. 2. Modified Hempel pipette.

open, the flow of mercury from the chamber into the trap is controlled with the stop-cock at the bottom of the Harington-Van Slyke chamber, until all except about 0.1 cc. of liquid has left the chamber. The liquid is then expelled from the trap.

Care is taken that the cup is free from water. With the tip of the  $\text{N NaOH}$  gas-free reagent container near the bottom of the cup, the stop-cock of the container is opened so that the liquid flows into the cup at a rate of about 1 cc. in 2 seconds. When the cup is nearly filled (and while the liquid is still flowing from the con-



tainer) about 2 cc. of the solution are run quickly into the chamber by controlling the reaction chamber stop-cock. When the cup fills again, the reagent container is removed.

The 2 cc. of washing solution have now drained to the bottom of the chamber and are promptly removed into the trap. About 4 cc. of additional liquid are admitted from the cup, leaving at least 1 cc. in the cup as a protective layer against absorption of air.

The stop-cock is sealed with mercury and the chamber is shaken for about 15 seconds. This shaking should be so vigorous that the liquid splashes effectively against the walls of the reaction chamber, and all of the precipitated iodine is removed. During this shaking with the leveling-bulb stop-cock open, and the stop-cock at the bottom of the reaction chamber closed, the leveling bulb is lowered until the liquid in the trap from the previous washing is drawn down to the bottom of the trap so that all of the precipitated iodine on the walls of the trap is effectively removed. The motor is stopped and the liquid in the trap, together with any supernatant gas, is expelled. The liquid in the chamber is then drawn into the trap, leaving about 0.1 cc. of liquid in the chamber, so as surely not to draw any gas from the chamber. The liquid in the trap is drawn down to the bottom of the trap and then expelled. The stop-cock at the bottom of the chamber is finally left open to the chamber.

*Absorption of Nitric Oxide with  $\text{KMnO}_4$* —6 cc. (or more) of the phosphate-nitrate-permanganate solution are introduced into the cup and 3 cc. are admitted at once into the chamber. The chamber is shaken slowly with the motor for about 10 seconds, and as the gas is absorbed, the mercury rises in the chamber. An additional cc. of  $\text{KMnO}_4$  is added and the chamber is shaken for another 10 seconds, while the volume of the gas is so reduced that the aqueous meniscus enters the constricted portion of the chamber near the 2 cc. mark. The remaining 2 cc. of  $\text{KMnO}_4$  are added gradually in about 1 minute in about four 0.5 cc. instalments. Agitation is obtained by raising the leveling bulb gradually about 15 cm. above the lower ring support, and then letting it down suddenly to the lower ring support. Care must be taken not to lower the leveling bulb below the lower ring support, so as not to draw dissolved air out of the permanganate solution; and not to raise the leveling bulb above the level of the mercury in the

chamber, so as not to lose gas through the stop-cock at the top of the chamber. The rate of addition of the permanganate and the method of agitation may be varied greatly without sacrificing accuracy. It is important to make analyses and blanks in the same manner. If the absorption of nitric oxide is not complete after 6 cc. of  $\text{KMnO}_4$  have been added, one should add 1 or 2 cc. of additional permanganate from the cup. Unless unusually large amounts of  $\text{NO}$  are present (more than 30 cc. at 1 atmosphere), 6 cc. of  $\text{KMnO}_4$  are ample to effect complete absorption.

The cup is washed several times with water until free from permanganate color, and about 2.5 cc. of gas-free  $\text{N NaOH}$  are introduced into the cup, 2 cc. of which are admitted to the chamber. The stop-cock is sealed with mercury.

*Measuring the Nitrogen Gas*—After  $\frac{1}{2}$  minute (to allow for complete absorption of  $\text{CO}_2$  and  $\text{N}_2\text{O}$ ) the aqueous meniscus is brought exactly to either the 0.5 or the 2.0 cc. mark and the manometer reading,  $p_1$ , together with the temperature, is recorded. It is important that this step be carried out with a minimum of agitation in order that as little as possible of the dissolved air will be extracted from the permanganate solution.

The gas is expelled with minimal loss of liquid (as much as 0.2 cc. of liquid loss does no harm). After sealing the stop-cock with mercury, the aqueous meniscus is again set at the 0.5 or 2.0 cc. mark and the manometer reading,  $p_0$ , is recorded.

For washing the chamber between analyses, the use of dilute nitric acid or  $\text{NaNO}_2$  and acetic acid is very effective. The nitrous acid must be completely removed by at least three washings with water before the next amine sample is introduced.

After many analyses a yellow solid sometimes collects in the trap below the reaction chamber, which is not removed by ordinary washing of the chamber. The following washing method has been found effective in removing this solid.

After rinsing the chamber with  $\text{NaNO}_2$  and acetic acid, followed by water, add about 5 cc. of saturated  $\text{Na}_2\text{S}_2\text{O}_3$  solution to the chamber, followed by about 50 cc. of water. With both stop-cocks of the chamber and the stop-cock to the leveling bulb open, the leveling bulb is gradually and *cautiously* lowered so that the water in the chamber flows into the glass tubing below the reaction chamber, until it reaches the 4-way junction above the leveling bulb

stop-cock, when the leveling bulb stop-cock is closed. Care must be taken to avoid lowering the leveling bulb so rapidly that the water enters the manometer tube. A suction tube leading to the waste bottle is attached to the glass tubing immediately above the stop-cock which is above the 4-way junction. This stop-cock is then gradually and cautiously opened until the water meniscus in the chamber falls to the 50 cc. mark. (Care must be taken not to open this stop-cock so suddenly that a considerable amount of mercury is drawn out of the manometer, so that water later would be sucked into the manometer.) The leveling bulb is placed in the upper ring support and the stop-cock leading to the leveling bulb is gradually opened, until the mercury has flowed into the reaction chamber to about the 50 cc. mark, and continuity to the mercury in the manometer is established. Then the stop-cock above the 4-way junction is opened until the water is completely displaced with mercury. The water is expelled from the reaction chamber and the chamber is washed twice with water.

The blank analysis of reagents and calculations are the same as the description given by Van Slyke ((4) pp. 434-437).

#### *Method with Harington-Van Slyke Chamber without KI*

The procedure is the same as that with KI, except that glacial acetic acid without KI is used.

#### *Construction and Use of Modified Hempel Pipette*

In our experience with Van Slyke's form of Hempel pipette, small amounts of gas occasionally become trapped in the capillary adjacent to the stop-cock. This occurs usually when grease collects in and around the bore of the stop-cock and especially in old pipettes when after extensive use small grooves or nicks appear on the core of the stop-cock. This difficulty can be entirely avoided by a pipette in which there is no stop-cock in the delivery capillary, and in which the stop-cock controlling the flow of gas is in the tube between the two reservoirs (see Fig. 2). This pipette is also less expensive and in our experience less apt to be broken. The details in the use of this pipette will now be described.

*Transfer of NO + N<sub>2</sub> Gas Mixture to Permanganate Pipette and Absorption of NO*—After the amine-nitrous acid reaction is complete, the stop-cock leading to the leveling bulb is opened and the

leveling bulb is allowed to remain in the lower ring support. About 1 cc. of gas-free water<sup>3</sup> is introduced into the cup above the chamber. By momentary opening of the reaction chamber stop-cock to the cup, the mercury in the capillaries is displaced with gas-free water. (If this mercury were not removed, it would later be driven into the  $\text{KMnO}_4$  pipette, which is to be avoided.)

The tip of the pipette (see Fig. 2) is introduced into the cup, and by opening the pipette stop-cock about 6 cc. of  $\text{KMnO}_4$  solution are allowed to flow from the pipette into the cup while the tip of the pipette is held below the liquid level. The pipette tip is then pressed and held firmly into the bottom of the cup, while its stop-cock is allowed to remain open wide. The leveling bulb of the gas apparatus is placed in the upper ring support, and after the gas in the chamber is under greater than atmospheric pressure, the leveling bulb stop-cock is closed. The reaction chamber stop-cock is then opened wide, and by controlling the leveling bulb stop-cock, the  $\text{N}_2 + \text{NO}$  mixture is caused to flow into the pipette. As soon as the first visible portion of nitrous acid solution enters the pipette capillary, the leveling bulb stop-cock is closed, and the reaction chamber stop-cock and the pipette stop-cock are also closed. The leveling bulb is placed in the lower ring support. In blood filtrate analyses it is important that not more than 0.03 cc. of the iodine-nitrous acid solution enter the Hempel pipette capillary.

While it is held with firm pressure in the bottom of the cup, the pipette is shaken with a horizontal back and forth rotation for about 5 seconds (not longer), which promptly creates a vacuum in the pipette. The shaking is stopped and the pipette stop-cock is opened until the  $\text{KMnO}_4$  solution from the pipette reservoir has restored the gas pressure. The pipette stop-cock is closed and a second and third shaking of 10 seconds each and restoration of pressure are effected. After a third (or fourth) 10 second shaking, when a small amount of gas still remains to be absorbed, the gas pressure is restored by lifting the pipette tip from the bottom of the cup, so that the pipette capillary is filled with  $\text{KMnO}_4$  solution from the cup. Here it is important to watch the level of the permanganate in the cup, and if it falls to as low as 2 cm. from the

<sup>3</sup> Distilled water is boiled and drawn while hot into a gas-free reagent container over mercury (see Fig. 1), where it is stored.

bottom of the cup, the flow of liquid is stopped by pressing the tip again into the bottom of the cup; in this case the pressure is finally restored by opening the pipette stop-cock. Usually after the fourth shaking the volume of gas remaining to be absorbed is less than 3 or 4 cc., and after the pipette tip is once lifted from the bottom of the cup at this stage, it is not necessary to press it down again. With the tip lifted from the bottom of the cup, and while still immersed in the  $\text{KMnO}_4$  solution in the cup, the pipette is shaken a final 30 seconds to insure complete absorption of  $\text{NO}$ . The pipette is then removed from the cup and supported with the tip immersed in a test-tube containing water.

In this manipulation the duration of the first and second shakings should not be longer than 5 and 10 seconds respectively in order that the vacuum will not draw appreciable amounts of dissolved air from the permanganate solution. If the gas is all absorbed before the pipette tip is lifted (as may inadvertently happen when one opens the stop-cock instead of lifting the pipette after the fourth shaking), one fills the pipette capillary with  $\text{KMnO}_4$  solution, by applying gentle suction with the mouth through a rubber tube attached to the open end of the pipette reservoir, and momentary opening of the pipette stop-cock, while the lifted pipette tip is immersed in the  $\text{KMnO}_4$  solution in the cup.

Any one of several other manipulations may be used to effect the absorption of the  $\text{NO}$  in the pipette; for example, the pipette stop-cock may be left open throughout the absorption, and the pipette capillary finally filled with  $\text{KMnO}_4$  solution from the cup by suction applied to the open end of the pipette reservoir.

*Return of Purified  $\text{N}_2$  to Manometric Chamber*—After the chamber is washed and charged with about 10 cc. of gas-free water, and 1 cc. of this water is returned to the cup, the cup is nearly filled with ordinary water. With the leveling bulb stop-cock open, the leveling bulb is raised to the upper ring support until there is no evacuated space in the manometer. The leveling bulb stop-cock is closed and the leveling bulb placed in the lower ring support.

The tip of the Hempel pipette is introduced below the surface of the water in the cup and held there, while the pipette stop-cock is carefully opened until most of the permanganate has flowed out of the pipette capillary and the permanganate- $\text{N}_2$  meniscus is

about 2 cm. from the tip. The tip of the pipette is pressed and held firmly in the bottom of the cup and the pipette stop-cock and also the reaction chamber stop-cock are opened wide. By controlling the leveling bulb stop-cock, the gas in the pipette is caused to flow into the reaction chamber and, as soon as the first permanganate following the gas is seen to enter the capillaries of the reaction chamber stop-cock, the leveling bulb stop-cock is closed. The other two stop-cocks are then closed and the pipette is removed.

The cup is washed until free from permanganate color and about 0.2 cc. of water is drawn into the chamber from the cup in order to displace the permanganate from the capillaries at the base of the cup and of the reaction chamber stop-cock. The stop-cock is then sealed with mercury. We have found it desirable to use a minimal amount of mercury here for, as the mercury droplets fall through the gas and water phases, they carry bubbles of gas down to the mercury-water surface, which at times has a significant lowering effect on the subsequent pressure reading. This is all the more important since in setting the meniscus in preparation for this reading, all agitation must be avoided in order to prevent the solution of the  $N_2$  in the water. The washing with about 0.2 cc. of water immediately before sealing the stop-cock with mercury renders the use of all except minimal quantities of mercury unnecessary.

#### SUMMARY

By the use of potassium iodide in the reaction mixture, Van Slyke's manometric amino nitrogen method has been modified to yield theoretical values with cystine and glycine, as well as with other common amino acids. A simplified form of Hempel pipette is described, which we feel makes the method more reliable.

A method is described in which the entire analysis is carried out in the Harington-Van Slyke chamber, and which thus avoids the use of the Hempel pipette. This method is recommended as the most convenient and uniformly reliable of the manometric amino nitrogen methods.

With blood filtrates (after correction for urea) the KI methods yield from 8 to 15 per cent less  $N_2$  than Van Slyke's method, indicating that blood probably contains significant amounts of sub-

stances like cystine and glycine, which yield too high values for amino nitrogen by Van Slyke's method.

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## THE STEROLS OF SILKWORM FECES\*

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Schoenheimer (1) has shown that higher organized animals do not utilize the phytosterols of their vegetable diet but excrete them with the feces. Plant sterols have not yet been found in tissues of higher animals. There are some indications, however, that lower forms of animal life behave differently towards the sterols of their plant diet. The principal sterol of certain vegetarian mollusks for instance is a derivative of sitosterol (2), and sitosterol itself has been found in appreciable quantities in the fat of the chrysalis of *Bombyx mori* (3). The latter case is of special interest because cholesterol and sitosterol occur simultaneously at a ratio of about 5:1. Owing to the lack of controlled feeding experiments, it remains undecided whether the sitosterol is of endogenous or exogenous character.

In order to obtain some preliminary information concerning this question the author has attempted to isolate and identify the sterols present in the droppings of the silkworm. The sterols were obtained from the non-saponifiable fraction of the ether extract of dried feces by way of the digitonides. The crude sterols, melting at 127–128°, were acetylated and the acetates, m.p. 124° and  $[\alpha]_D = -38.3^\circ$ , brominated in ether and glacial acetic acid. On standing in the cold a slight precipitate had been formed, which after one recrystallization showed a melting point of 118–119°. It was identical with dibromocholesteryl acetate. The presence of small amounts of cholesterol in feces of different origin has been frequently demonstrated (4).

The soluble bromides were then debrominated with sodium

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† Textile Foundation, Inc., Silk Research Fellow, 1936–37.



iodide in alcohol (5). The sterol acetate so obtained shows after several recrystallizations a melting point of  $125^{\circ}$  and  $[\alpha]_D = -38.4^{\circ}$ . The sterol itself melted at  $129-130^{\circ}$ , optical rotation  $[\alpha]_D = -33.52^{\circ}$ .

The amount of unsaturated sterol present in the sterol was determined by Schoenheimer's method (6). It was found to be about 9 to 13 per cent.

Though the melting point of the principal sterol of the feces is somewhat low, there can be little doubt that we have to deal with ordinary sitosterol. The depression of the melting point is probably due to the presence of saturated sterols and small amounts of cholesterol. A similar observation was made by Schoenheimer, who found that the melting point of the principal sterol of rabbit feces was  $128.5^{\circ}$  (7).

#### EXPERIMENTAL

The dried feces which had been ground to a fine powder in a ball mill were extracted in a Soxhlet apparatus first with ether for 72 hours and then with chloroform for 120 hours. The ether extract and the ether-soluble part of the chloroform extract combined represented 5.2 per cent of the dried feces. To a solution of 5 gm. of the dark green wax-like material a solution of 2 gm. of sodium in 75 cc. of absolute alcohol was added. After the mixture had stood for 72 hours, ether and water were added and the aqueous layer was drawn off. The latter was washed several times with ether. The combined ether extracts were then washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness. 1.92 gm. of non-saponifiable material were obtained, which correspond to 38.5 per cent of the ether extract.

The non-saponifiable material was taken up with 50 cc. of boiling 60 per cent alcohol. A considerable amount of substance, consisting mainly of straight chain hydrocarbons and alcohols of the order  $C_{30}-C_{34}$  remained undissolved. To the filtrate 100 cc. of a 1 per cent digitonide solution were added. After 24 hours the precipitate which had been formed was filtered off, washed carefully with alcohol and ether, and dried *in vacuo* at  $100^{\circ}$ . 780 mg. of digitonides were so obtained, representing 195 mg. of sterol.

The sterols have also been prepared from the non-saponifiable fraction obtained by the saponification of the ether extract with hot alcoholic potassium hydroxide. In one instance the non-saponifiable material was first separated into an alcoholic and a non-alcoholic fraction by way of the potassium salts of the esters of sulfonic acid (8). These were then hydrolyzed with alcoholic sulfuric acid according to a method recommended by Butenandt and Westphal (9) to give a mixture of sterols and higher straight chain alcohols. The latter are sufficiently insoluble in cold ether to permit their partial separation. From the mother liquors the sterols were obtained by way of the digitonides.

From the digitonides obtained by the different methods the sterols were recovered with the help of Schoenheimer's method (10). In all instances sterols of identical properties were obtained. The yields were from 0.16 to 0.20 per cent of the dried feces.

The sterols, which showed a melting point of  $127-128^{\circ}$ , were acetylated in boiling acetic anhydride. The acetates after three recrystallizations from ether and methanol showed a melting point of  $124^{\circ}$ .

*Rotation*—0.1010 gm. of substance dissolved in chloroform and made up to 3 cc. gave in a 1 dm. tube a reading of  $\alpha = -1.288^{\circ}$ ; hence  $[\alpha]_D^{24} = -38.29^{\circ}$ .

500 mg. of the acetate were dissolved in 5 cc. of dry ether. The solution to which 6 cc. of glacial acetic acid containing 5 per cent of bromine had been added was kept at  $0^{\circ}$  for 8 hours. The small amount of precipitate which had been formed after that time was filtered off, washed, and dried. It weighed 12 mg. After one recrystallization from ether and alcohol the bromide showed a melting point of  $117-118^{\circ}$ . When mixed with dibromocholesteryl acetate of the same melting point, it melted at  $117^{\circ}$ .

The filtrate containing the soluble bromide was mixed with a solution of 1 gm. of sodium iodide in an excess of ethyl alcohol and refluxed for 1 hour (5). After being cooled, the brown solution was poured into an aqueous solution of sodium sulfite. The debrominated material was then extracted with ether, and the extract washed with water, sodium bicarbonate solution, and water. The acetate obtained from the extract was recrystallized twice from ether and alcohol. The melting point was then  $125^{\circ}$ .

*Rotation*—0.1033 gm. of substance dissolved in chloroform and made up to 3 cc. gave in a 1 dm. tube a reading of  $\alpha = -1.35^\circ$ ; hence  $[\alpha]_D^{24} = -38.32^\circ$ .

The sterol obtained by saponification of the acetate melted after several recrystallizations from alcohol at  $129-130^\circ$ . The melting point could not be raised by further recrystallizations.

*Rotation*—63.9 mg. of substance dissolved in chloroform and made up to 3 cc. gave in a 1 dm. tube a reading of  $\alpha = -0.74^\circ$ ; hence  $[\alpha]_D^{25} = -33.51^\circ$ .

The determination of the saturated sterols present was carried out under close observation of the directions given by Schoenheimer (6). 135.3 mg. and 60.4 mg. gave 68.4 mg. and 22 mg. of digitonide or 12.6 and 9.1 per cent of saturated sterol. Owing to the small amount of digitonide the nature of the saturated sterol could not be determined.

#### SUMMARY

From the feces of the silkworm, *Bombyx mori*, a mixture of sterols has been isolated which was found to consist of a small amount of cholesterol and a sitosterol-like compound with a melting point of  $129-130^\circ$  and  $[\alpha]_D = -33.52^\circ$ . The sitosterol contained from 9 to 13 per cent of saturated sterols. The feces sterols of *Bombyx mori* show great similarity to those of other vegetarian animals, e.g. the rabbit.

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## CONFIGURATIONAL RELATIONSHIPS OF THE ALIPHATIC AND AROMATIC AMINO ACIDS

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(Received for publication, October 13, 1936)

The configurational relationships of most of the aliphatic amino acids and likewise of the aromatic amino acids have been established by direct chemical methods. There remained to establish the configurational relationship between the aromatic and aliphatic amino acids. This task has now been accomplished by the oxidation of *l*-tyrosine to *l*-aspartic acid. Inasmuch as the configuration of the levo-1-phenyl-2-aminomethane has been correlated by Leithe<sup>1</sup> to *l*-(dextro)alanine, the correlation of the configuration of tyrosine to the amine is likewise established. It remains to convert tyrosine into 1-phenyl-3-aminopropane in order that the directions of rotation of the two configurationally related bases may also be established. Work in this direction is in progress.

The configurational relationships are presented in the accompanying set of formulæ.

The procedure by which aspartic acid was derived from tyrosine is similar to that employed by Leithe in the work mentioned above; namely, *N*-benzoyl-*l*-tyrosine ester was oxidized with chromic acid. The product of oxidation contained the *N*-benzoylaspartic acid. This was hydrolyzed and from the hydrolysis product aspartic acid was isolated as the mercury salt, which was then converted into the free acid.

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<sup>1</sup> Leithe, W., *Ber. chem. Ges.*, **64**, 2827 (1931).



|                      |             |                           |
|----------------------|-------------|---------------------------|
| $C_{11}H_{16}O_2NCl$ | Calculated. | C 53.74, H 6.57, Cl 14.43 |
| (245.58)             | Found.      | " 53.62, " 6.62, " 14.40  |

<sup>2</sup> Bergmann, M., Ulpts, R., and Camacho, F., *Ber. chem. Ges.*, **55**, 2796 (1922).

water and the mixture was allowed to stand overnight in a refrigerator. The crystalline deposit was filtered and recrystallized twice from benzene.

The substance had the following composition.

8.090 mg. substance: 0.314 cc. N<sub>2</sub> at 32° and  $p = 757.5$  mm.

4.114 " " : 10.395 mg. CO<sub>2</sub> and 2.320 mg. H<sub>2</sub>O

C<sub>11</sub>H<sub>19</sub>O<sub>4</sub>N. Calculated. C 68.97, H 6.11, N 4.47

(313 15) Found. " 68.90, " 6.19, " 4.33

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{-0.86^\circ \times 100}{3.7 \times 1} = -23.2^\circ \text{ (in alcohol)}$$

*l*-Aspartic Acid on Oxidation of the Above Product—7.0 gm. of the above material were dissolved in 100 gm. of glacial acetic acid and the solution was added slowly to 1 liter of glacial acetic acid containing 49 gm. of CrO<sub>3</sub>. The solution was heated for 3½ hours on the water bath in a flask provided with a return condenser. The excess of chromic acid was reduced by SO<sub>2</sub> and 100 cc. of water were added to the solution which was then concentrated under reduced pressure until most of the acetic acid was removed. To the aqueous solution kept in a cooling mixture 40.0 gm. of sulfuric acid were added gradually with constant stirring. The cold acid solution was exhaustively extracted with ether and from the extract the ether was removed in the usual manner. In a preliminary experiment, from this fraction a small portion of a substance was obtained with a composition approaching that of the N-benzoylmonoethyl ester of aspartic acid. The found values were C 59.63, H 6.05, and N 5.97, against the required C 58.86, H 5.66, and N 5.27. In the present experiment the entire fraction was taken up in 20 cc. of concentrated hydrochloric acid (sp. gr. 1.19) and refluxed for 4 hours on a boiling water bath. The benzoic acid which formed during the hydrolysis was removed by extraction with petroleum ether and the aqueous fraction concentrated to dryness under reduced pressure. The residue was taken up in water, the hydrochloric acid was removed from the solution by means of silver sulfate, and the excess of silver was removed by means of hydrogen sulfide and the sulfuric acid quantitatively by means of barium hydroxide. The final filtrate was concentrated

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to a crystalline mass, but, inasmuch as the material was not free of mineral matter, it was dissolved in water and the aspartic acid precipitated with mercuric acetate. The salt was freed from mercury ions by means of hydrogen sulfide and the filtrate on concentration formed a crystalline deposit having the composition and optical activity of *L*-aspartic acid.

5.109 mg. substance: 6.645 mg. CO<sub>2</sub>, 2.402 mg. H<sub>2</sub>O, 0.025 mg. ash

4.520 " " : 0.406 cc. N<sub>2</sub> at 26°, *p* = 755 mm.

C<sub>4</sub>H<sub>7</sub>O<sub>4</sub>N. Calculated. C 36.07, H 5.30, N 10.52

(133.06) Found. " 35.63, " 5.28, " 10.23

The rotation was

$$[\alpha]_{D^{20}}^{20} = \frac{+0.94^{\circ} \times 100}{3.85 \times 1} = +24.41^{\circ} (\pm 0.06)$$

## A NOTE ON THE STABILITY AND QUANTITATIVE DETERMINATION OF PHOSPHATIDES

By EVELYN B. MAN

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(Received for publication, October 8, 1936)

Reinvestigation of the method used in this laboratory for the determination of serum phosphatides proved that these substances are more unstable than has been thought previously. The method (7) had consisted of extraction of blood serum with alcohol and ether, evaporation of the lipid solvents, and, after sulfuric acid oxidation of organic material, determination by a modification of the Fiske and Subbarow method of all the phosphorus which had been dissolved by alcohol and ether. The reinvestigation of this method was prompted by the criticism of Page, Kirk, Lewis, Thompson, and Van Slyke (10) who found that, after evaporation of the alcohol and ether, reextraction of the residue by petroleum ether gave results for lipoid phosphorus 20 to 30 per cent lower than the alcohol-ether-soluble phosphorus. However, Kirk, Page, and Van Slyke (4) stated that in the petroleum ether extract even lower yields were obtained if the alcohol and ether were evaporated at 75° rather than at 60° or less. It seemed possible that decomposition of the phosphatides might occur even during evaporation of the alcohol and ether at temperatures of 60° or less when the evaporation was conducted in air at atmospheric pressure. For this reason, the phosphorus contents of the original alcohol-ether extract and of the petroleum ether solution of the alcohol-ether residue have been compared when evaporation of the alcohol and ether was carried out in the presence of an inert gas, at diminished pressures, and at a temperature not exceeding 37°.

### EXPERIMENTAL

Alcohol-ether-soluble phosphorus (Extract I), petroleum ether-soluble phosphorus (Extract II), and petroleum ether-insoluble,



alcohol-ether-soluble phosphorus (Extract III) have been determined in blood serum of seven different subjects. Data presenting the averages of duplicate determinations are given in Table I.

The alcohol-ether extraction was carried out in the manner previously described (6, 7). Equal aliquots of this extract were used for determination of the alcohol-ether-soluble phosphorus and for fractionation of the phosphorus into petroleum ether-soluble and petroleum ether-insoluble phosphorus. For fractionation the evaporation of the alcohol-ether was carried out under nitrogen at diminished pressure (approximately 28 inches of

TABLE I

*Yields of Phosphatide Phosphorus Obtained by Various Methods of Extraction of Blood Serum*

| Experiment No. | Extract I<br>Alcohol-ether-<br>soluble P | Extract II<br>Petroleum ether-<br>soluble, alcohol-<br>ether-soluble P | Extract III<br>Petroleum ether-<br>insoluble,<br>alcohol-ether-<br>soluble P | Alcohol-ether-<br>soluble P<br>redissolved in<br>petroleum ether |
|----------------|--|--|--|--|
|                | mg. per cent                             | mg. per cent   | mg. per cent   | per cent   |
| 1              | 13.70                                    | 13.00  | 0.47   | 95   |
| 2              | 11.27                                    | 11.17  | 0  | 99   |
| 3              | 8.92                                     | 8.47   | 0.41   | 95   |
| 4              | 38.70                                    | 38.40  | 0.31   | 99   |
| 5              | 10.76                                    | 10.41  | 0.44   | 97   |
| 6              | 52.40                                    | 52.30*   | 0.30   | 99.8   |
| 7              | 12.06                                    | 12.12  | 0.13   | 100.5  |

\* Extraction by the method of Kirk, Page, and Van Slyke (4) gave 50.8 mg. per cent of petroleum ether-soluble, alcohol-ether-soluble phosphorus.

mercury, the maximum water pump vacuum) with the temperature of the water bath ranging from 20–37°. Evaporation of the 8 cc. of alcohol-ether took 20 to 30 minutes. The instant that evaporation of the alcohol and ether was complete, petroleum ether was added to the residue. This solution was then centrifuged and the petroleum ether solution siphoned from any undissolved residue. Centrifugation was repeated twice, making in all three extractions with petroleum ether of the alcohol-ether residue. These steps were carried out as rapidly as possible and the final extraction with petroleum ether was always completed within 8 hours of the time when the blood had been taken.

After evaporation of the petroleum ether, digestion of the phos-

phatides with sulfuric and nitric acids and superoxol and estimation of the phosphorus by a modification of the Fiske and Subbarow method were carried out as originally described (7). In the case of the petroleum ether-insoluble, alcohol-ether-soluble phosphorus it was necessary to add 0.5 cc. of the phosphate standard in order to produce a color sufficiently intense to be read in the colorimeter. Calculation of this phosphorus fraction was made by difference. Special Pyrex centrifuge tubes were used throughout for the fractionation of the petroleum ether-soluble and petroleum ether-insoluble phosphorus. Two constrictions were blown into the walls of the tube to diminish the tendency of the solution to boil up the sides of the tube. The lowest bulb of the tube was constricted to contain 10 cc. and permit accurate dilution for the final colorimetric determination.

### *Results*

In each of the seven experiments 95 to 100.5 per cent of alcohol-ether-soluble phosphorus was recovered as lipid phosphorus soluble in petroleum ether, which means that only from 0 to 5 per cent of the phosphorus dissolved by alcohol and ether was insoluble in petroleum ether and therefore non-lipoid in nature. The maximum error of the Man and Peters method for the determination of lipid phosphorus was stated originally to be 5.0 per cent (7) and was found later to be less than this (8). Statistical analysis of 100 consecutive lipid phosphorus determinations gave a correlation between duplicates of 0.983, which means that the probable error of estimate of one of two duplicate determinations from the other is  $\pm 0.29$  mg. per cent. The extent to which the petroleum ether-soluble phosphorus falls short of the alcohol-ether-soluble phosphorus exceeds this slightly but definitely.

### DISCUSSION

When Le Breton (5), May (9), and Artom (1) found that 10 to 20 per cent of the alcohol-ether-soluble phosphorus was non-lipoid in nature, the substance examined for phosphatide content was the residue after evaporation of alcohol and ether in air. Although these workers used diminished pressure and low temperatures, the temperature was not stated and no mention was made of the use of an inert gas. That decomposition of the phosphatides occurs

during evaporation of alcohol and ether in air at low temperatures is in accordance with the observation of Kirk, Page, and Van Slyke (4) that lower yields of phosphatide obtained when the alcohol and ether were evaporated at 75° than when the alcohol and ether were evaporated at 60°. In one of our experiments alcohol and ether were evaporated from test-tubes open to the air and heated in a water bath several hours. The petroleum ether-soluble phosphorus was only about 50 per cent of the alcohol-ether-soluble phosphorus obtained from the residue after the alcohol and ether were evaporated with all the precautions described in the experimental method.

It is probable that the decomposition of the phosphatides which occurs when alcohol and ether are evaporated in air at 60° or less is related more closely to the action of the oxygen in the air than to the temperature. It was shown in the description of the Man and Peters (7) lipid phosphorus method that the sum of the lipid phosphorus and acid-soluble phosphorus agreed within experimental errors with the total phosphorus of blood serum. If phosphatides decompose at the temperature of boiling alcohol, extraction of the serum in refluxing alcohol and ether should have diminished the value of lipid phosphorus to such an extent that the total phosphorus would have exceeded the sum of the alcohol-ether-soluble phosphorus and the acid-soluble phosphorus. That the phosphatides do not decompose when heated to the temperature of boiling water was also demonstrated by Le Breton (5).

In selection of a method for the evaluation of phosphatides there has been much controversy as to the relative merits of two procedures. The method of estimating phosphatides from the quantity of lipid phosphorus was criticized by Bloor because of the 20 per cent of the alcohol-ether-soluble phosphorus which Le Breton had called non-lipoid in nature. The above data indicate that this criticism is not valid and greater accuracy is not attained by re-extraction with petroleum ether. The acetone precipitation of phosphatides which has been used by Bloor (2), Bloor and Snider (3), and Sinclair (11, 12) is subject to the criticism that before such acetone precipitation the original alcohol-ether solution of the phosphatides has been evaporated at atmospheric pressure in the presence of air. It is therefore reasonable to suppose that Bloor (2) and Sinclair (11) lost some of the phosphatides by decomposi-

tion during the process of evaporation in air before acetone precipitation.

Dr. Van Slyke and Dr. Page have kindly permitted us to mention that they, in a short series of experiments, have verified the observation that practically all of the phosphorus dissolved from blood serum by alcohol-ether is soluble also in petroleum ether (13), after removal of the alcohol and ether in absence of oxygen.

#### SUMMARY

Seven experiments have been presented which demonstrate that 95 to 100 per cent of the alcohol-ether-soluble phosphorus of blood serum is soluble also in petroleum ether, and may therefore be considered to be lipid in nature. Quantitative determination of phosphatides by determination of the total alcohol-ether-soluble phosphorus is therefore justified.

The approximately complete petroleum ether solubility of the phosphorus of the alcohol-ether residue was preserved only when the alcohol-ether was removed with precautions against the effects of heat and oxidation. The evaporation of the alcohol-ether was carried out at low pressure and temperature, and in a nitrogen atmosphere. That such precautions are necessary affords additional evidence of the instability of some of the phosphatides.

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## ON PROTEOLYTIC ENZYMES

### XII. REGARDING THE SPECIFICITY OF AMINOPEPTIDASE AND CARBOXYPEPTIDASE. A NEW TYPE OF ENZYME IN THE INTESTINAL TRACT

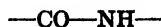
BY MAX BERGMANN AND JOSEPH S. FRUTON

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

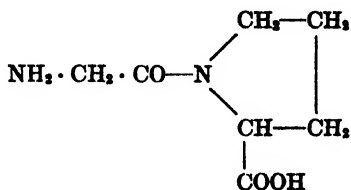
(Received for publication, October 16, 1936)

#### *Structural Specificity of Aminopeptidase*

In proteins there occur two different types of peptide linkages. The peptide linkages which preponderate are those containing a hydrogen atom ("peptide hydrogen"), as shown in Formula I. The peptide bonds in which the nitrogen atom of proline or hydroxyproline is involved differ from the general type in that they contain no peptide hydrogen and their nitrogen is heterocyclic. This latter type of peptide linkage may be illustrated by means of glycylproline (Formula II).



I



II

In collagen and gelatin more than one-quarter of all the peptide linkages correspond to the second type. For the understanding of the digestion and metabolism of proteins it is necessary to learn how the peptide linkages of the second type are affected by various proteolytic enzymes.

Up to the present time only *one* enzyme has been found (1) which was capable of splitting glycyl-*l*-proline and *l*-alanyl-*l*-

proline;<sup>1</sup> namely, a crude preparation of aminopeptidase. The enzyme was prepared from intestinal erepsin by removal of the dipeptidase by adsorption on iron hydroxide (2). The aminopeptidase prepared in this manner is not a homogeneous substance and it was not clear whether the splitting of glycylproline was due to the aminopeptidase itself or to another hitherto unknown enzyme in the intestinal mucosa. In the experiments reported in this paper an attempt was made to clarify this question in the following manner. The specificity of aminopeptidase is such that it splits *l*-leucylglycylglycine or *l*-alanylglycylglycine at the pep-

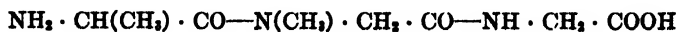
TABLE I  
*Hydrolysis of Peptides by Extract of Intestinal Mucosa*

| Substrate                              | Time        | Hydrolysis      |
|--|-------------|-----------------|
|  | <i>hrs.</i> | <i>per cent</i> |
| <i>dl</i> -Leucylglycylglycine         | 2           | 104*            |
| <i>l</i> -Alanylglycylglycine          | 2           | 117†            |
| <i>l</i> -Alanylsarcosylglycine        | 6           | 0               |
|  | 24          | 1               |
| Glycyl- <i>l</i> -proline              | 6           | 75              |
| Glycyl- <i>dl</i> -proline             | 23          | 84*             |
|  | 72          | 96*             |
| Glycylsarcosine                        | 21          | 2               |
| Carbobenzoxylglycyl- <i>l</i> -proline | 22          | 0               |

\* Per cent of the *l* component.

† Owing to the presence of dipeptidase the hydrolysis does not stop at 100 per cent.

tide linkage adjacent to the free amino group. We first investigated (Table I) the behavior of aminopeptidase toward *l*-alanylsarcosylglycine (Formula III).



### III

This peptide lacks a peptide hydrogen in the peptide linkage adjacent to the free amino group, as in the case of glycylproline.

<sup>1</sup> In a previous communication (1) this substance was called *d*-alanyl-*l*-proline, according to the older nomenclature.

The behavior of this tripeptide toward aminopeptidase should show whether the enzyme is capable of splitting peptide linkages without the peptide hydrogen. Furthermore, we compared the effects of various inhibitors on the splitting of leucylglycylglycine by aminopeptidase and on the splitting of glycylproline by an extract of intestinal mucosa.

All the experiments reported in Table I were carried out with the same enzyme extract. The rapid splitting of leucylglycylglycine and *l*-alanylglycylglycine indicates that the enzyme solution was rich in aminopeptidase. However, in contrast to *l*-alanylglycylglycine, *l*-alanylsarcosylglycine was not attacked at all, even after an extended period of time. Thus aminopeptidase requires a peptide hydrogen for its action; therefore, it cannot be the component of intestinal mucosa which is responsible for the splitting of glycyl-*l*-proline. There must be another enzyme present in the intestinal mucosa which is able to split glycyl-*l*-proline. We should like to call this enzyme *prolidase* in order to indicate that in the substrates of this enzyme the proline nitrogen is present as imido nitrogen. In contrast to *prolidase*, the *prolinase* of Grassmann (3) splits substrates such as prolylglycine in which the proline nitrogen is present as imino nitrogen.

It will be noted that *prolidase* does not split carbobenzoxyglycyl-*l*-proline. The substitution of the free amino group thus inhibits the enzyme action. Furthermore, the antipodal specificity of *prolidase* was investigated by subjecting glycyl-*dl*-proline to the action of crepsin. The fact that only the *l* component was split demonstrates the antipodal selectivity of the enzyme with respect to the proline component. The resistance of glycyl-*d*-proline to ereptic action is a further argument for the presence of the enzyme *prolidase* distinct from aminopeptidase, since the antipodal specificity of the latter enzyme is determined only by the amino acid having the free amino group.

Glycylsarcosine is similar to glycylproline in the fact that it is a dipeptide lacking a peptide hydrogen. However, in contrast to glycylproline, glycylsarcosine is not split by *prolidase* (Table I). Thus *prolidase* cannot simply be considered a dipeptidase which does not require peptide hydrogen but rather seems to be specifically adapted to the splitting of peptide linkages containing *proline* nitrogen. This restriction may be due to the fact that the



proline nitrogen and  $\alpha$ -carbon are linked in a ring system, thus forcing this part of the molecule into a definite spatial arrangement.

The experiments reported in Table II show that the splitting of leucylglycine by dipeptidase and the splitting of leucylglycylglycine and alanylglycylglycine by aminopeptidase is almost com-

TABLE II  
*Inhibition of Ereptic Enzymes by HCN*

| Substrate              | Time | Hydrolysis |          |
|------------------------|------|------------|----------|
|                        |      | No HCN     | M/12 HCN |
|                        | hrs. | per cent   | per cent |
| Glycyl-l-proline       | 4    | 68         | 52       |
| dl-Leucylglycylglycine | 2    | 101        | 5        |
| l-Alanylglycylglycine  | 2    | >100       | 8        |
| dl-Leucylglycine       | 2    | 96         | 2        |

TABLE III  
*Inhibition of Peptidases by Phenylhydrazine*

| Enzyme           | Substrate                    | Time | Hydrolysis         |                 |                  |                   |
|------------------|------------------------------|------|--------------------|-----------------|------------------|-------------------|
|                  |                              |      | No phenylhydrazine | 0.04 mm per cc. | 0.004 mm per cc. | 0.0004 mm per cc. |
|                  |                              | min. | per cent           | per cent        | per cent         | per cent          |
| Prolidase*       | Glycyl-l-proline             | 180  | 59                 | 50              |                  |                   |
| Dipeptidase*     | dl-Leucylglycine             | 80   | 41                 | 6               | 14               | 28                |
|                  |                              | 180  | 65                 | 8               | 27               | 34                |
| Aminopeptidase*  | l-Alanylglycylglycine        | 80   | 94                 | 84              |                  | 94                |
|                  |                              | 180  | 98                 | 86              |                  | 98                |
|                  | dl-Leucylglycylglycine       | 80   | 98                 | 84              | 90               |                   |
|                  |                              | 180  | 107                | 88              | 92               |                   |
| Carboxypeptidase | Carbobenzoxyglycyl-l-alanine | 150  | 49                 | 56              |                  |                   |

\* Extract of intestinal mucosa.

pletely inhibited by M/12 cyanide solution. However, the splitting of glycylproline is affected only slightly, indicating that the action of prolidase may be observed under conditions in which dipeptidase and aminopeptidase are suppressed.

Von Euler and Josephson (4) found that dipeptidase action is

inhibited by phenylhydrazine. It was recently found in our laboratory that in papain, liver cathepsin, and bromelin there are enzymes the action of which is similarly inhibited by phenylhydrazine. It was of interest to learn the behavior of prolidase toward phenylhydrazine. Table III shows that prolidase, as well as aminopeptidase, is only slightly inhibited by phenylhydrazine, while carboxypeptidase is not inhibited at all.

### *Antipodal Specificity of Aminopeptidase*

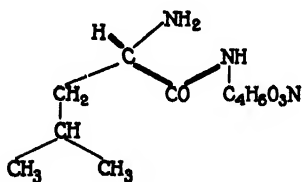
Aminopeptidase splits *l*-leucylglycylglycine to *l*-leucine and glycylglycine, but is completely ineffective upon *d*-leucylglycylglycine. Aminopeptidase, therefore, shows definite antipodal specificity and it was found that this specificity applies only to the amino acid which bears the free amino group of the substrate.

We have recently attempted to correlate the antipodal specificities of various enzymes (5, 6) by means of a general theory which might be designated a "polyaffinity" theory. This approach may be summarized as follows: An enzyme exhibiting antipodal specificity must contain several active groups in a fixed asymmetric arrangement. During enzymatic action these active groups enter into combination with a similar number of active groups of the substrate and force the latter into a fixed spatial position. In this combination one might imagine a plane determined by the active groups of the enzyme (binding plane of the enzyme) and similarly a plane determined by the active groups of the substrate (binding plane of the substrate). The enzyme-substrate combination (and the enzymatic splitting of the substrate) is only possible if the enzyme and substrate can approach each other in such a way that the two hypothetical binding planes are only a few Ångström units apart.

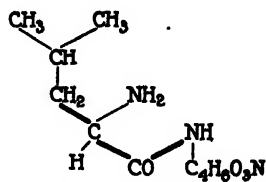
The polyaffinity theory may be applied to aminopeptidase and the splitting of leucylglycylglycine in the following manner. The active groups in the substrate for aminopeptidase are the free amino group and various atomic groups of the adjacent peptide linkage. The binding plane of the *l*-leucylglycylglycine may therefore be determined, as in Formula IV, by the successive groups NH, CO,  $\alpha$ -carbon atom, and NH<sub>2</sub>.<sup>2</sup> The  $\alpha$ -carbon atom

<sup>2</sup> The fact that the peptide linkage may exist in the form  $\text{—C(OH)—N—}$  is disregarded for the present (5).

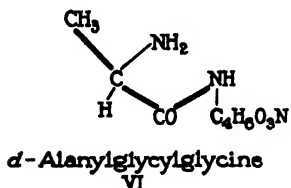
is not an active group but lies in the binding plane. On one side of the plane there juts out the hydrogen atom, on the other side the voluminous isobutyl group. It is immediately evident that aminopeptidase can approach the active groups of *l*-leucylglycylglycine only from the side on which the small hydrogen atom is situated, and where the enzyme finds the groups NH, CO,  $\alpha$ -carbon atom, and NH<sub>2</sub> arranged in a clockwise order. On the basis of the polyaffinity theory, one may say that aminopeptidase is capable of splitting only those substrates which it is able to approach from the side exhibiting the above arrangement of the



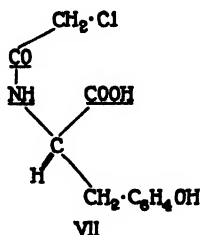
*l*-Leucylglycylglycine  
IV



*d*-Leucylglycylglycine  
V



*d*-Alanylglycylglycine  
VI



VII

active groups. In the case of *d*-leucylglycylglycine (Formula V) this side of the potential binding plane is not approachable for the enzyme because of the presence of the voluminous isobutyl group.

If in Formula V the isobutyl group is replaced by the much smaller methyl group—that is, when *d*-alanylglycylglycine (Formula VI) is subjected to the action of aminopeptidase—then the polyaffinity theory permits us to predict that the approach of the enzyme would be made more difficult but yet not prevented completely. *d*-Alanylglycylglycine should therefore be split much

more slowly than the *l* antipode, but nevertheless at a measurable rate. We have tested this requirement of the polyaffinity theory experimentally and have found it to be confirmed (Table IV).

TABLE IV  
*Hydrolysis of d- and l-Alanylglycylglycine by Intestinal Mucosa*

| Substrate                     | Time | Hydrolysis |
|-------------------------------|------|------------|
|                               | hrs. | per cent   |
| <i>l</i> -Alanylglycylglycine | 2    | 117        |
| <i>d</i> -Alanylglycylglycine | 2    | 10         |
|                               | 7    | 15         |
|                               | 24   | 20         |
|                               | 68   | 28         |

TABLE V  
*Hydrolysis of Peptides by Carboxypeptidase*

| Substrate                             | Time | Hydrolysis                  |                          |
|---------------------------------------|------|-----------------------------|--------------------------|
|                                       |      | Without addition of gelatin | With addition of gelatin |
|                                       | hrs. | per cent                    | per cent                 |
| Chloroacetyl- <i>l</i> -tyrosine      | 0.25 | 37*                         |                          |
|                                       | 0.50 |                             | 37*                      |
| Carbobenzoxyglycyl- <i>l</i> -alanine | 7    | 105                         |                          |
|                                       | 3    |                             | 93                       |
| Carbobenzoxyglycyl- <i>d</i> -alanine | 7    | 9                           |                          |
|                                       | 19†  | 13                          |                          |
|                                       | 6    |                             | 13                       |
|                                       | 23†  |                             | 18                       |
| <i>dl</i> -Leucylglycine              | 2.5  | 1                           |                          |
|                                       | 23   | 1                           |                          |
| <i>dl</i> -Leucylglycylglycine        | 2.5  | 2                           |                          |
|                                       | 23   | 1                           |                          |

\* Tyrosine crystals had already separated out.

† After this reaction period the enzyme lost its activity under the conditions of the experiment.

### *Antipodal Specificity of Carboxypeptidase*

In substrates for carboxypeptidase the active groups are an  $\alpha$ -carboxyl group and the atoms of the adjacent peptide linkage. The enzyme shows antipodal specificity with respect to the amino

acid which bears the  $\alpha$ -carboxyl group and the asymmetric carbon of which thus lies between the active groups. This specificity may be interpreted in the same manner as in the case of aminopeptidase and dipeptidase; namely, on the basis of a polyaffinity relationship between enzyme and substrate. Since carboxypeptidase splits chloroacetyl-*L*-tyrosine (Formula VII) one may conclude that the enzyme splits only those substrates in which the groups  $\text{CO}\cdot\text{NH}\cdot\text{CH}(\text{R})\cdot\text{COOH}$  are arranged in a counter-clockwise order and in which the  $\alpha$ -hydrogen atom of the CHR group is directed toward the enzyme.

This requirement is fulfilled when the free carboxyl belongs to an *L*-amino acid. The polyaffinity theory leads to the expectation that substrates in which the amino acid bearing the carboxyl is *D*-alanine would be split by carboxypeptidase, but more slowly than the *L* antipode.

In order to test the theory experimentally we studied the splitting of both antipodes of carbobenzoxyglycylalanine by carboxypeptidase. The crystalline enzyme, prepared according to Anson (7) was employed. The results given in Table V conform to the requirements of the polyaffinity theory.

#### DISCUSSION

The identification of prolidase as a distinct enzyme has clarified the problem of the aminopeptidase. It is now evident that aminopeptidase, like dipeptidase and carboxypeptidase, requires in its substrates the presence of a peptide hydrogen. The question which then arises is whether these three peptidases react, within the limits of their different specificities, with their substrates according to some uniform general mechanism. The general view, as expressed in repeated discussions of this question, has been that dipeptidase and carboxypeptidase react with their substrates according to fundamentally different mechanisms. Waldschmidt-Leitz (8) expressed the view that dipeptidase and aminopeptidase condense with the amino groups of their substrates with the formation of Schiff bases, but that carboxypeptidase reacts with its substrate through an ionic mechanism. The basis of this hypothesis is the important observation of von Euler and Josephson which was mentioned previously—that phenylhydrazine inhibits the action of erepsin on dipeptides. The

results presented in Table III indicate, however, that only dipeptidase is strongly inhibited by phenylhydrazine; aminopeptidase, carboxypeptidase, and prolidase are not thus inhibited. Furthermore, Bergmann and Ross (9) showed recently that the splitting of carbobenzoxytriglycine by HCN-papain is completely inhibited by phenylhydrazine. Carbobenzoxytriglycine contains no free amino group and therefore offers no opportunity for the formation of a Schiff base. It must be concluded from these observations that one cannot interpret the inhibition of dipeptidase by phenylhydrazine as indicative of a condensation of dipeptidase with its substrate to form a Schiff base. Furthermore, there is no basis for considering dipeptidase and aminopeptidase as being similar enzymes fundamentally different from carboxypeptidase.

Another principle of classification of proteolytic enzymes, which has been widely employed up to the present time, differentiates the enzymes according to the molecular weight of their substrates. Dipeptidase, aminopeptidase, carboxypeptidase, and prolinase were designated peptidases because they split peptides of low molecular weight in contrast to pepsin, trypsin, papain, cathepsin, and bromelin which were assumed to split only proteins and high molecular protein degradation products (8, 10). Recently, however, there have been found in papain, cathepsin, bromelin, and in tryptic proteinase partial enzymes which hydrolyze low molecular synthetic substrates. All these enzymes split peptide linkages and are therefore peptidases; however, in contrast to dipeptidase, aminopeptidase, and carboxypeptidase, they are not restricted to the terminal peptide linkages of their substrates but preferably attack more central peptide bonds. Therefore, the concept of peptidases must be extended to include these enzymes, and the peptidases may be separated into two classes, one of which is restricted to terminal peptide linkages (exopeptidases) and the other, which is capable of attacking central peptide linkages as well (endopeptidases) (9). Since all enzymes which attack native proteins split off many different polypeptides from the large protein molecule, these enzymes must belong to the group of endopeptidases.

A further basis for the classification of peptidases is offered by the nature of the active groups of their substrates. All peptidases, the specificities of which have been investigated, require, in

addition to the peptide linkage to be split, one or more active groups in the substrate. They differ according to the nature and number of these active groups. Finally, many peptidases require the presence of peptide hydrogen in the peptide linkage to be split, while other peptidases do not. On the basis of these considerations the accompanying classification may be suggested.

*Classification of Peptidases*

| Endopeptidases*          | Exopeptidases                             |                                       |  |
|--------------------------|---|---------------------------------------|--|
|                          | Split peptide bonds with peptide hydrogen |                                       | Split peptide bonds without peptide hydrogen |
|                          | With one additional active group          | With several additional active groups |  |
| Papainpeptidase I and II | Aminopeptidase                            | Dipeptidase                           | Prolidase                                    |
| Cathepsin I and II       | Carboxypeptidase                          | Prolinase                             |  |
| Bromelin I " II          |   |                                       |  |
| Pepsin                   |   |                                       |  |
| Trypsin                  |   |                                       |  |

\* Since several of the endopeptidases have been shown to be two enzyme systems with special activation properties, it would be possible to subdivide the endopeptidases on this basis. Another principle of classification, based on the structural specificity of the endopeptidases, is made available by the finding of synthetic substrates for several endopeptidases.

### EXPERIMENTAL

#### *l-Alanylesarcosylglycine*

*Carbobenzoxy-l-Alanylesarcosylglycine*—To a solution of 5 gm. of sarcosylglycine in 10 cc. of saturated potassium bicarbonate solution there was added, in small portions with shaking, an ethereal solution of carbobenzoxy-*l*-alanyl chloride (1) prepared from 4 gm. of carbobenzoxy-*l*-alanine. After removal of the ether, the aqueous solution was acidified to Congo red and extracted three times with ethyl acetate. The combined extracts were evaporated down, and, upon the addition of ether, the resulting syrup crystallized in needles. Yield, 2.6 gm. M.p., 108°.

|                      |             |                       |
|----------------------|-------------|-----------------------|
| $C_{16}H_{21}N_3O_6$ | Calculated. | C 54.7, H 6.0, N 11.9 |
| 351.2                | Found.      | " 54.5, " 6.0, " 11.9 |

*Free Tripeptide*—1.2 gm. of the carbobenzoxytripeptide were hydrogenated in methyl alcoholic solution with palladium as the catalyst. On evaporating the solution, the substance crystallized in needles. Yield, 0.5 gm.

$C_8H_{17}O_4N_3 + 1H_2O$ . Calculated. C 40.9, H 7.3, N 17.8,  $H_2O$  7.6  
 235.2 Found. " 40.9, " 7.3, " 17.6, " 7.5  
 $[\alpha]_D^{25} = +10.8^\circ$  (5% in water)

### *d-Alanlylglycylglycine*

*Carbobenzoxy-d-Alanlylglycine Hydrazide*—3.3 gm. of carbobenzoxy-*d*-alanlylglycine ethyl ester (5) were dissolved in 15 cc. of absolute alcohol and 1.3 cc. of hydrazine hydrate were added. After standing overnight at room temperature, ether was added to yield 2.7 gm. of the substance. After recrystallization from water the melting point was 147–148°.

$C_{13}H_{18}O_4N_4$ . Calculated. C 53.0, H 6.2, N 19.0  
 294.2 Found. " 53.0, " 6.1, " 19.3

*Carbobenzoxy-d-Alanlylglycylglycine Benzyl Ester*—To a solution of 2 gm. of the above hydrazide in 25 cc. of water, 4 cc. of glacial acetic acid, and 1 cc. of concentrated hydrochloric acid there was added, within 2 to 3 minutes, with cooling and shaking, a solution of 1.2 gm. of sodium nitrite in 7 cc. of water. After the resulting syrup was extracted with ether, the ether layer was washed with cold water, twice with bicarbonate, and again with cold water, passed through a dry filter, and treated with a dry ether solution of glycine benzyl ester prepared from 6 gm. of the hydrochloride. A crystalline precipitate formed immediately, which was filtered after standing several hours at 0°. Yield, 3.7 gm. M.p., 116°.

$C_{22}H_{28}N_2O_6$ . Calculated. C 61.8, H 5.9, N 9.8  
 427.2 Found. " 61.7, " 6.0, " 9.8

*Free Tripeptide*—2 gm. of the carbobenzoxytripeptide benzyl ester were hydrogenated in the usual manner in methyl alcohol containing 0.35 cc. of glacial acetic acid. On evaporation of the



filtrate the substance crystallized out in needles. Yield, 0.9 gm. The substance was recrystallized from water-alcohol.

$C_7H_{11}O_4N_2 + 1H_2O$ . Calculated. C 38.1, H 6.8, N 19.0,  $H_2O$  8.1  
221.1 Found. " 38.3, " 6.7, " 19.2, " 7.9  
 $[\alpha]_D^{25} = -31.6^\circ$  (10% in water)<sup>3</sup>

*l-Alanylglycylglycine*

*Carbobenzoxy-l-Alanylglycine Hydrazide*—This was prepared in the same manner as the *d* form. M.p., 145–147°.

$C_{11}H_{18}O_4N_4$ . Calculated. C 53.0, H 6.2, N 19.0  
294.2 Found. " 53.0, " 6.1, " 19.2

*Carbobenzoxy-l-Alanylglycylglycine Benzyl Ester*—This was prepared in the same manner as the *d* form. M.p., 114–116°.

$C_{22}H_{28}N_2O_6$ . Calculated. C 61.8, H 5.9, N 9.8  
427.2 Found. " 61.6, " 5.9, " 9.8

*Free Tripeptide*—This was prepared in the same manner as the *d* form.

$C_7H_{11}O_4N_2 + 1H_2O$ . Calculated. C 38.1, H 6.8, N 19.0,  $H_2O$  8.1  
221.1 Found. " 38.1, " 6.7, " 19.0, " 7.9  
 $[\alpha]_D^{25} = +32.4^\circ$  (10% in water)<sup>4</sup>

*Glycyl-dl-Proline*

*Carbobenzoxyglycyl-dl-Proline*—This substance was prepared from *dl*-proline in the same manner as the *l* form (1). M.p., 129–130°.

$C_{11}H_{18}O_3N_2$ . Calculated. C 58.8, H 5.9, N 9.1  
306.2 Found. " 58.9, " 6.1, " 9.3

*Free Dipeptide*—This substance was prepared in the same manner as the *l* form.

$C_7H_{11}O_2N_2$ . Calculated. C 48.8, H 7.0, N 16.3  
172.1 Found. " 48.6, " 7.3, " 16.1  
 $[\alpha]_D^{25} = -0.1^\circ$  (2% in water)

<sup>3</sup> Fischer (11) gives  $[\alpha]_D^{25} = -29.4^\circ$ .

<sup>4</sup> Fischer (12) gives  $[\alpha]_D^{25} = +31.4^\circ$ .

*Carbobenzoxylglycyl-l-Alanine*—This was prepared according to Abderhalden and Neumann (13). M.p., 135°.

|                        |                   |                         |
|------------------------|-------------------|-------------------------|
| $C_{13}H_{16}O_5N_2$ . | Calculated.       | C 55.7, H 5.7, N 10.0   |
| 280.2                  | Found.            | " 55.5, " 5.7, " 10.1   |
|                        | $[\alpha]_D^{25}$ | = -9.5° (5% in alcohol) |

Abderhalden and Neumann (13) report a melting point of 155–156° and  $[\alpha]_D^{20}$  = -4.7°. Our calculation of the rotation from the experimental data of Abderhalden and Neumann gives the value  $[\alpha]_D^{20}$  = -6.4°. The correctness of our data for carbobenzoxylglycyl-l-alanine is supported by the fact that the *d* isomer, described below, has the same melting point and magnitude of rotation.

*Carbobenzoxylglycyl-d-Alanine*—This was prepared in the same manner as the *l* form. M.p., 135°.

|                        |                   |                         |
|------------------------|-------------------|-------------------------|
| $C_{13}H_{16}O_5N_2$ . | Calculated.       | C 55.7, H 5.7, N 10.0   |
| 280.2                  | Found.            | " 55.7, " 5.7, " 10.3   |
|                        | $[\alpha]_D^{25}$ | = +9.3° (5% in alcohol) |

*Glycylsarcosine*—This was prepared as described in (1).

*Glycyl-l-Proline*—This was prepared as described in (1).

### *Enzymatic Studies*

In the experiments reported above, the substrate concentration was kept at 0.005 mm per cc. In the case of *dl*-peptides, 0.1 mm was employed and the splitting was calculated only for the natural form. The pH of the reaction mixture was maintained at the optimum for each enzyme with the appropriate *m*/3 phosphate buffer. The temperature in all cases was 40°.

The extent of hydrolysis was determined by titration of carboxyl groups in 90 per cent alcohol with 0.01 *N* KOH, thymolphthalein being used as the indicator (14). 100 per cent splitting represents an increase of 1 cc. in the titration of 0.2 cc. of the reaction mixture.

The erepsin solution was prepared by extracting the intestinal mucosa with 87 per cent glycerol for 2 days, the resulting extract being diluted with an equal volume of water. A clear solution was obtained by centrifugation.

The crystalline carboxypeptidase was prepared from pancreatic juice according to Anson (7).

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## THE ISOLATION OF A NEW POLYSACCHARIDE SYNTHESIZED BY A SOIL MICROORGANISM

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Recent investigations of several of the polysaccharides obtained by the agency of microorganisms have contributed towards the knowledge of the structure of the more complex carbohydrates. The work of Haworth, Raistrick, and Stacey (1, 2) and others who have prepared complex polysaccharides by the action of microorganisms on nutrient solutions containing glucose, mannose, and other sugars suggests that a similar mechanism of polysaccharide synthesis may operate in the plant.

Microorganisms are not only capable of synthesizing polysaccharides from simple sugars, but also of transforming simple sugars into their isomers. For example, the growth of *Penicillium charlesii* on glucose produces a polysaccharide composed of mannose units (1); on the other hand, the microorganism investigated by the writers produces a polysaccharide consisting of glucose residues when grown on culture solutions containing mannitol. These examples will serve to demonstrate the manifold character of the polysaccharides which may exist, and also suggest that, of the numerous polysaccharides which occur in nature, only a comparatively small number have been isolated and characterized.

The microorganism which produces the polysaccharide described in this paper was isolated by Dr. C. B. Lipman from an old mud brick. This organism, when grown in culture medium containing 0.2 per cent mannitol, produced a viscous colloidal solution from which a white flocculent precipitate separated when poured into an excess of ethyl alcohol. This crude polysaccharide, when reprecipitated several times from ethyl alcohol, did not reduce Fehling's solution, did not give a coloration with iodine, and was free of nitrogen. Its specific rotation,  $[\alpha]_D$ , was  $+140^\circ$ . Hydrol-

ysis with 2 per cent sulfuric acid yielded 96.5 per cent glucose. From the product of hydrolysis a glucosazone with a melting point of  $208^{\circ}$  and a glucose *p*-bromophenylosazone with a melting point at  $216^{\circ}$  were prepared. No sugars other than glucose could be found in the product of hydrolysis.

Acetylation of the polysaccharide yielded a triacetate with a specific rotation of  $[\alpha]_D = +148^{\circ}$  in chloroform. The polysaccharide could be regenerated from its triacetate and possessed practically the same constants as before acetylation.

The reducing power of the polysaccharide, determined according to Bergmann and Machemer (3), gave an iodine value of 2.5. The molecular weight of the triacetate determined by the method of Rast (4) was 2765, and when determined by the viscosity method of Staudinger and Nodzu (5) was equal to 2980. These values show the polysaccharide to be made up of approximately nine or ten anhydroglucose residues.

#### EXPERIMENTAL

*Description of the Microorganism*—The organism which produced the polysaccharide is a non-spore-bearing bacterium measuring about  $3.6\mu$  by  $0.9\mu$ . It was obtained by Dr. C. B. Lipman from a mud brick taken from a wall in an old Roman village which was built about 400 A.D. in the western desert of Egypt.

The bacillus was found in a mannitol culture prepared for nitrogen-fixing bacteria. The composition of the medium was as follows:

|   |         |   |         |
|---|---------|---|---------|
| Mannitol.....                                 | 2.0 gm. | FeCl <sub>3</sub> .....                   | Trace   |
| K <sub>2</sub> HPO <sub>4</sub> .....         | 0.5 "   | MnSO <sub>4</sub> ·4H <sub>2</sub> O..... | "       |
| MgSO <sub>4</sub> ·7H <sub>2</sub> O. . . . . | 0.2 "   | CaCO <sub>3</sub> .....                   | 10 gm.  |
| NaCl.....                                     | 0.2 "   | Distilled water.....                      | 1 liter |

The organism appeared in the crude culture as a medium sized rod with an extremely large capsule, about  $5\mu$  by  $8\mu$ , which stained lightly with saturated alcoholic Gentian violet. Colonies grown on agar did not show the capsulation as clearly as in the crude cultures. In solution cultures of the single organism the capsular material dissolved off into the medium, forming a viscous colloidal mass. The organism was unable to fix atmospheric nitrogen, but was able to grow in the above medium in which the

only sources of nitrogen were the impurities in the chemicals and traces of fixed nitrogen from the air.

*Isolation of the Polysaccharide*—1 liter flasks containing the medium were inoculated with the organism. The cultures were grown 2 to 4 weeks and then evaporated on a steam bath to a small volume. The calcium carbonate and other solid particles were centrifuged off, and the supernatant liquid was poured into about 10 times its volume of 95 per cent ethyl alcohol. A precipitate then separated in the form of a white flocculent mass. This mass was filtered off, dissolved in water, and reprecipitated five times by dissolving in water and pouring into 70 per cent alcohol. The precipitate was finally washed with 95 per cent alcohol, then with ether, and dried in the vacuum oven at 60°. 10 liters of the culture solution yielded about 1 gm. of the substance. This substance did not reduce Fehling's solution, was free of nitrogen, and its ash content was 0.8 per cent.

*Specific Rotation*— $[\alpha]_D = +140^\circ$  (in water,  $c = 0.4$ )

*Analysis*— $(C_6H_{10}O_5)_n$ . Calculated. C 44.4, H 6.1  
Found. " 43.9, " 6.3

*Hydrolysis*—A solution of 2 gm. of the dry polysaccharide in 100 cc. of 2 per cent sulfuric acid was heated under a reflux condenser at a temperature of 105–110° for 8 hours, cooled, nearly neutralized with hot saturated solution of barium hydroxide, and completely neutralized with barium carbonate. The mixture was then heated to 80° and allowed to stand overnight, filtered, the precipitate washed with hot water, and the filtrate diluted to 200 cc. The reducing power of this solution was determined by the Munson and Walker procedure (6). The yield of reducing sugars calculated as glucose was 96.5 per cent of that required for a theoretical yield.

*Identification of Glucose*—From the hydrolyzed solution two osazones were prepared: first, a phenylosazone by the method of Mulliken (7), which had a melting point of 208°; second, a *p*-bromophenylosazone by the method of van der Haar (8), with a melting point of 216°. These melting points correspond to those of the respective osazones of glucose. The osazones were compared under the microscope with glucosazone and glucose *p*-bromophenylosazone prepared from pure glucose. They proved to be identical.

The product of hydrolysis was examined for mannose, fructose, galactose, and pentose sugars. None of these sugars could be detected. The polysaccharide also gave a negative test for uronic acids.

*Acetylation*—The polysaccharide displayed resistance to acetylation when the Haworth, Hirst, and Woolgar method (9) of treatment with pyridine and acetic anhydride was used. Barnett's method, as modified by Haworth, Hirst, and Webb (10), with chlorine and sulfur dioxide as catalysts was therefore employed. The yield of the triacetate was 78.3 per cent of that of the theoretical.

*Specific Rotation*— $[\alpha]_D = +148^\circ$  (in chloroform,  $c = 0.5$ )  
*Analysis*— $(C_6H_7O_5(CH_2CO)_3)_n$ . Calculated.  $CH_2CO$  44.8  
Found. " 44.7

*Regeneration of the Polysaccharide from Its Triacetate*—The acetate was deacetylated by allowing it to stand for 24 hours at room temperature with 50 per cent aqueous methyl alcoholic potassium hydroxide. After acidifying with acetic acid, 2 volumes of 95 per cent alcohol were added, and the resulting precipitate washed with 80 per cent alcohol and reprecipitated from water by the addition of 2 volumes of alcohol.

The properties of the regenerated polysaccharide appeared little altered, except that its opacity when dissolved in water was less than that of the original polysaccharide.

*Specific Rotation*— $[\alpha]_D = +138^\circ$  (in water,  $c = 0.4$ )

*Determination of Iodine Number*—The reducing value of the polysaccharide was investigated according to the method of Bergmann and Machemer (3). The iodine number (number of cc. of 0.1 N I required to oxidize 1 gm. of the substance) was found to be 2.5. Those given by Haworth and Percival (11) for starch and glycogen are 0.7 and 1.95. While the iodine number does not express the exact molecular weight of a polysaccharide, it shows its relative reducing power and therefore reflects the relative length of the chain. If we compare the iodine number, 2.5, of the isolated polysaccharide with the value of 1.95 for glycogen given by Haworth and Percival, the length of the chain of the anhydroglucose residues of the polysaccharide is somewhat lower than that of glycogen.

*Estimation of Molecular Weight*—The molecular weight of the triacetate was determined by the Rast method (4) and by the viscosity method of Staudinger and Nodzu (5). These determinations showed the polysaccharide to consist of nine or ten anhydroglucose residues.

*Molecular Weight*—2765 (Rast); 2980 (Staudinger)

#### SUMMARY

A polysaccharide synthesized by a soil microorganism was isolated from a culture containing 0.2 per cent mannitol. This polysaccharide was shown to consist of approximately nine or ten anhydroglucose units.

The authors are indebted to Dr. C. B. Lipman for his advice in the course of this investigation.

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## THE ACTIVITY OF GLYCINE IN AQUEOUS SOLUTION AT TWENTY-FIVE DEGREES\*

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Precise measurements of the thermodynamic activities of pure amino acids in aqueous solution over a wide concentration range have not been made except by the freezing point method (1, 4, 6, 8, 18, 19); the activities calculated thence are valid only at or near 0°. A recently described isopiestic method (17, 20) permits accurate measurements of the activity of water in a solution with a non-volatile solute relative to that in a solution of known activity. This method has been used to determine the activity of water in glycine solutions at 25°, and from these data the osmotic and the activity coefficients of glycine have been calculated.

### *Procedure*

**Materials**—The sucrose was a standard sample purchased from the Bureau of Standards. The glycine was a commercial product. It was divided into two parts, one being recrystallized twice from a mixture of alcohol and water, and the other recrystallized twice from water alone. Moisture determinations on the glycine were made by drying over magnesium perchlorate at 105°.

No significant differences were observed between the results obtained with the two samples of glycine.

**Apparatus**<sup>1</sup>—Four cylindrical dishes, with simple hinge covers,

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† Honorary Research Fellow in Physiology, 1933-37.

‡ Emerson Fellow in Pharmacology and Toxicology, 1934-36.

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each with a capacity of about 20 cc. and weighing about 25 gm., were spun from pure silver. These dishes were fitted into depressions made in a round 12 pound copper block, which acted as a heat reservoir and conducting medium. The dishes and copper block were placed in a 6 inch desiccator. Short copper wires supporting the covers were so arranged that they might be pushed aside by a brass rod extending through the desiccator top, allowing the dishes to close by gravity without the desiccator being opened.

Approximately 2 cc. of an amino acid solution of known molality were placed in each of two dishes, and a sucrose solution of similar molality was placed in the other two dishes. The dishes were weighed to the nearest mg. before and after addition of the solution. The desiccator containing the dishes was evacuated by a water pump to about 25 mm. of mercury and placed in a motor-driven rocker in a constant temperature bath at 25° and rocked through an angle of 15° and return at the rate of 30 times a minute. The dishes of solution were removed and weighed every day. The criterion for equilibrium was the maintenance of a consistent molal ratio for 2 or more days. Equilibrium was attained in 1 or 2 days with concentrated solutions, but 4 to 5 days were sometimes required with dilute solutions. Several determinations over a moderate range of concentration could be made with the same solutions by the distillation *in vacuo* of part of the water from the solutions.

### Results

60 determinations were made with twelve solutions, the results of which are summarized in Table I. It was repeatedly demonstrated, in confirmation of the results of Robinson and Sinclair (17), that the concentrations of two dilute solutions (approximately 0.2 M) of sucrose having initial concentrations differing by 4 to 10 per cent became identical within  $\pm 0.3$  per cent after 4 days. The error with more concentrated solutions was usually less.

A large scale plot was made of  $m_B/m_A$  against  $m_B$  (Fig. 1). The variation of this ratio with  $m_B$  may be expressed by the equation

$$m_B/m_A = 1 - 0.204 m_B + 0.0538 m_B^2 - 0.00758 m_B^3 \quad (1)$$

**Reference Solutions**—The activity of water in a solution is determined by the isopiestic method only if the activity of the

water in the solution with which it is equilibrated is known. Robinson and Sinclair (17) used potassium chloride solutions for reference, but it seemed desirable to choose a non-electrolyte as a standard for amino acid solutions, since the latter have been shown to resemble non-electrolytes in their thermodynamic properties (21). Therefore, in this investigation sucrose solutions were

TABLE I

*Concentrations, in Moles per 1000 Gm. of Water, of Isopiestic Solutions of Sucrose and Glycine at 25°*

| <i>m<sub>S</sub></i> | <i>m<sub>A</sub></i> | <i>m<sub>S</sub></i> | <i>m<sub>A</sub></i> | <i>m<sub>S</sub></i> | <i>m<sub>A</sub></i> |
|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| 0.2112               | 0.2195               | 0.6297               | 0.7080               | 1.3814               | 1.7166               |
| 0.2214               | 0.2318               | 0.6512               | 0.7348               | 1.4840               | 1.8753               |
| 0.2272               | 0.2381               | 0.7047               | 0.8008               | 1.5047               | 1.9100               |
| 0.2357               | 0.2473               | 0.7120               | 0.8130               | 1.5446               | 1.9621               |
| 0.2428               | 0.2544               | 0.7168               | 0.8148               | 1.5538               | 1.9713               |
| 0.2599               | 0.2736               | 0.7508               | 0.8647               | 1.5776               | 2.0220               |
| 0.2954               | 0.3134               | 0.7671               | 0.8840               | 1.6729               | 2.1611               |
| 0.3193               | 0.3389               | 0.7996               | 0.9228               | 1.7209               | 2.2389               |
| 0.3491               | 0.3706               | 0.8285               | 0.9564               | 1.7710               | 2.3202               |
| 0.3664               | 0.3938               | 0.9179               | 1.0828               | 1.8348               | 2.4214               |
| 0.3734               | 0.4022               | 0.9415               | 1.1126               | 1.8481               | 2.4418               |
| 0.3912               | 0.4230               | 1.0422               | 1.2501               | 1.8710               | 2.4742               |
| 0.4023               | 0.4356               | 1.0608               | 1.2777               | 1.9126               | 2.5306               |
| 0.4090               | 0.4390               | 1.2206               | 1.4983               | 2.0437               | 2.7462               |
| 0.4178               | 0.4490               | 1.2296               | 1.5120               | 2.1179               | 2.8648               |
| 0.4345               | 0.4672               | 1.2310               | 1.5056               | 2.1701               | 2.2588               |
| 0.4471               | 0.4872               | 1.2598               | 1.5508               | 2.2456               | 3.0908               |
| 0.4930               | 0.5363               | 1.2745               | 1.5636               | 2.2848               | 3.1536               |
| 0.5354               | 0.5888               | 1.3043               | 1.6110               | 2.3438               | 3.2552               |
| 0.6120               | 0.6868               | 1.3691               | 1.7044               | 2.3722               | 3.3059               |

used as the reference standard.<sup>2</sup> The activity of the solvent in sucrose solutions was calculated from the freezing point measurements of Ewan (3), Loomis (9), Morse and Frazer (11), Raoult (14), and Rivett (15) by means of the equation ((7) p. 284)

$$\log a_1 = -0.004211\theta - 0.0000022\theta^2 \quad (2)$$

Similar calculations were made from the osmotic pressure measurements at 0° of Morse, Holland, Zies, Myers, Clark, and Gill (13)

<sup>2</sup> The use of sucrose as a reference substance was suggested by Dr. B. B. Owen of the Department of Chemistry.

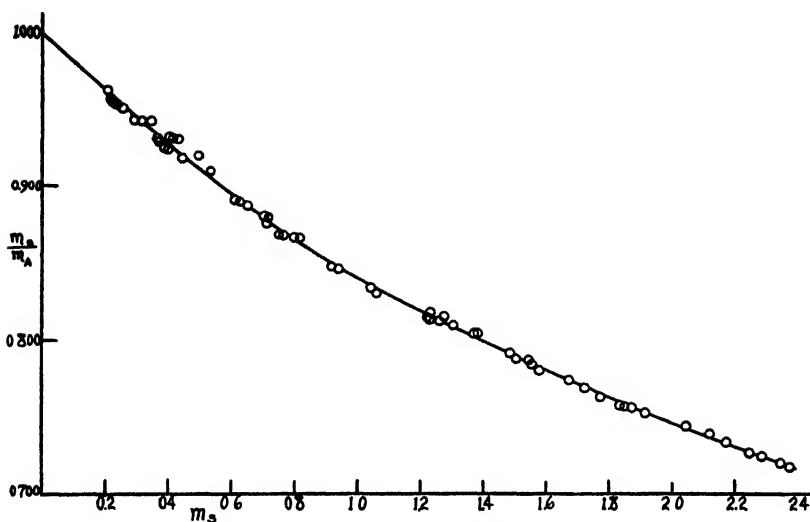


FIG. 1. Ratios of concentrations of isopiestic solutions of sucrose and glycine at 25°.

and of Berkeley, Hartley, and Burton (2), by the equation of Frazer and Myrick (5)

$$2.3026 \log \frac{p_1}{p_0} = -\frac{\pi V_0}{RT} \quad (3)$$

and from the vapor pressure measurements of Berkeley, Hartley, and Burton (2) by the equation ((7) p. 257)

$$\log a_1 = \log \frac{p_1}{p_0} \quad (4)$$

At 30° the activity of the solvent was calculated from the osmotic pressure measurements of Frazer and Myrick (5), Lotz and Frazer (10), and Morse, Holland, Myers, Cash, and Zinn (12) and from the vapor pressure measurements of Berkeley, Hartley, and Burton (2). Some other measurements of the colligative properties of sucrose solutions at various temperatures are recorded in the literature, but they are less extensive or less consistent than those mentioned.

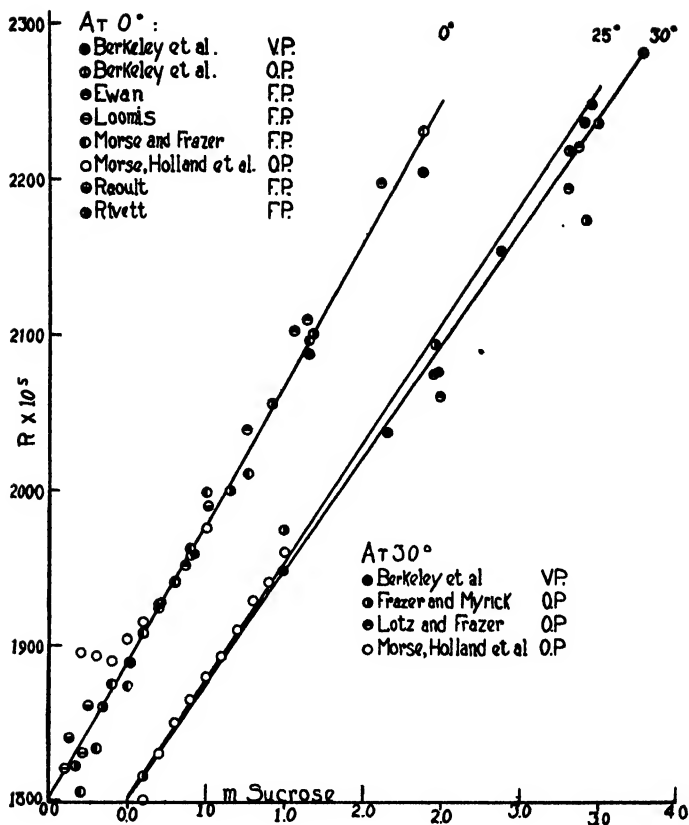


FIG. 2. The relative molal lowering of the vapor pressure of water in sucrose solutions at 0°, 30°, and, by interpolation, at 25°.

From each set of measurements values were calculated for the sensitive function (17)

$$R = \frac{1 - a_1}{m} = \frac{1 - \frac{p_1}{p_0}}{m} \quad (5)$$

This function was plotted against the molality. It was apparent, as shown in Fig. 2, that the results up to 3 m at 0° and 30° were best represented by straight lines passing through the theoretical limit, 0.018015. By linear interpolation from the slopes of these

lines a line was obtained for 25° (Fig. 2) which may be represented by the equation

$$R = 0.01802 + 0.00153m_s \quad (6)$$

Experiments in progress elsewhere<sup>3</sup> indicate that the empirical constant 0.00153 may be slightly smaller. It may be seen that  $R$  changes very little with temperature, the values chosen for 25° differing but little from the direct determinations at 30°.

TABLE II  
*Thermodynamic Properties of Sucrose Solutions at 25°*

| $m$ | $\varphi_s$ |
|-----|-------------|
| 0.1 | 1.009       |
| 0.2 | 1.019       |
| 0.3 | 1.029       |
| 0.4 | 1.038       |
| 0.5 | 1.047       |
| 0.7 | 1.066       |
| 1.0 | 1.096       |
| 1.2 | 1.116       |
| 1.5 | 1.145       |
| 2.0 | 1.196       |
| 2.5 | 1.247       |

From the values of  $R$  at 25° the osmotic coefficients  $\varphi_s$  were calculated (16) where

$$\varphi_s = -\frac{55.51}{m_s} 2.3026 \log a_1 = -\frac{127.817}{m_s} \log (1 - Rm_s) \quad (7)$$

These values are shown in Table II.

Since at equilibrium the activity of water in the sucrose and in the glycine solutions is the same, it may readily be shown from Equation 7 that the osmotic coefficients of the glycine solutions,  $\varphi_A$ , are determined by the relation

$$\varphi_A = \frac{m_s}{m_A} \varphi_s \quad (8)$$

<sup>3</sup> Personal communication from Professor George Scatchard of the Massachusetts Institute of Technology.

The values of  $\varphi_A$  calculated from Equations 1, 7, and 8 are shown in Table III.

The activity coefficients of the solute,  $\gamma_2$ , in a two component solution may be calculated from the activity of the solvent by the Gibbs-Duhem equation. Lewis and Randall ((7) p. 274) calculate

TABLE III

*Thermodynamic Properties of Glycine Solutions at 25°*

The results of Scatchard and Prentiss at 0° are shown for comparison.

| $m_A$ | $\varphi_{25^\circ}$ | $\varphi_{0^\circ}$ | $\gamma_{25^\circ}$ | $\gamma_{0^\circ}$ |
|-------|----------------------|---------------------|---------------------|--------------------|
| 0.1   | 0.990                | 0.989               | 0.980               | 0.978              |
| 0.2   | 0.981                | 0.978               | 0.962               | 0.957              |
| 0.3   | 0.973                | 0.968               | 0.946               | 0.937              |
| 0.4   | 0.965                | 0.958               | 0.930               | 0.918              |
| 0.5   | 0.957                | 0.949               | 0.915               | 0.900              |
| 0.7   | 0.944                | 0.932               | 0.888               | 0.868              |
| 1.0   | 0.928                | 0.910               | 0.854               | 0.826              |
| 1.2   | 0.921                | 0.898               | 0.838               | 0.802              |
| 1.5   | 0.913                | 0.884               | 0.816               | 0.771              |
| 1.7   | 0.908                | 0.876               | 0.801               | 0.754              |
| 2.0   | 0.903                | 0.869               | 0.786               | 0.733              |
| 2.5   | 0.894                |                     | 0.760               |                    |
| 3.0   | 0.888                |                     | 0.741               |                    |
| 3.3   | 0.885                |                     | 0.729               |                    |

molal activity coefficients for non-electrolytes by a modification of this equation

$$2.3026 \log \gamma_2 = -h - \int_0^m \frac{h}{m} dm \quad (9)$$

where 
$$h = \frac{55.51}{m} 2.3026 \log a_1 + 1 \quad (10)$$

Consideration of Equations 7 and 10 shows that the function  $h$  is equal to  $1 - \varphi$ . The integral may be estimated with sufficient accuracy from a plot of  $(1 - \varphi_A)/m$  against  $m$ . The activity coefficients calculated in this way are shown in Table III.

The most accurate and extensive freezing point measurements of glycine are probably those of Scatchard and Prentiss (19). Accurate osmotic and activity coefficients may be calculated from their



equations since the heat of dilution of glycine is small (21). These are shown in Table III for comparison. As might be expected glycine is a more nearly perfect solute in water at 25° than at 0°, but the change in activity with temperature is small.

Similar measurements of the other  $\alpha$ -amino acids are being made in these laboratories.

The authors thank Professor D. I. Hitchcock for his criticism and encouragement in this research.

#### SUMMARY

The osmotic coefficients and the activity coefficients of glycine in aqueous solution at 25° have been determined by an isopiestic method with sucrose as the reference substance.

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## THE CARBOHYDRATE METABOLISM OF BRAIN\*

### III. ON THE ORIGIN OF LACTIC ACID

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Holmes and Holmes (1925,a) found that the "reducing substances" of brain<sup>1</sup> were not capable of giving rise to lactic acid, and that less lactic acid was present in the brain of insulinized than in normal animals. They attributed the lowering of the "resting" lactic acid level not to any direct effect of insulin but to the lowered blood sugar (1925,b). The same authors could detect no significant rise of lactic acid nor decrease of glycogen during incubation of chopped brain, and suggested "that the brain depends upon the blood sugar, rather than on any substance which it stores itself, for lactic acid precursor" (1926). Further studies in 1927 (Holmes and Holmes) confirmed the dependence of brain lactic acid on the blood sugar level, the brains of depancreatized animals having a "resting" value corresponding to the degree of hyperglycemia at death.

Kinnersley and Peters (1929, 1930) were unable to account for raised levels of lactic acid in pigeon brain on the assumption that lactic acid originates from the sugar of blood, and concluded that it must arise from some form of carbohydrate in the brain itself. Jungmann and Kimmelstiel (1929) found that during a 2 hour period of autolysis the brain glycogen diminished while lactic acid increased, and concluded that the lactic acid originated from the glycogen.<sup>2</sup> In view of the fact that both Kinnersley and Peters

\* Aided by a grant from the Rockefeller Foundation.

<sup>1</sup> Analyzed several minutes after death.

<sup>2</sup> In view of the fact that glycogen (measured by the method of Kerr (1936)) disappears almost completely within 2 hours after death, the high value they obtained after autolysis indicates that their method was faulty. Moreover, there was no agreement between the amount of fall of glycogen and the rise of lactic acid.

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(1929, 1930) and Jungmann and Kimmelstiel (1929) questioned the conclusions of Holmes and Holmes (1925,b, 1926, 1927) regarding the precursor of lactic acid, Holmes and Sherif (1932) reexamined this question, attempting to secure initial values for lactic acid and carbohydrate closer to the true resting level. They found relatively little glycogen\* in the brain (33 to 60 mg. per 100 gm.), the amount being independent of the blood sugar level, and the fall on incubation was small compared with the total rise of lactic acid. The amount of lactic acid formed in the brain was found to depend on the blood sugar level. They concluded that "glycogen is not an intermediate in the conversion of glucose into lactic acid by the brain."

It is clear that in none of the studies mentioned above were true resting values obtained for either lactic acid or glycogen, for minimum lactic acid concentrations can be obtained only when the brain is frozen *in situ* (Avery, Kerr, and Ghantus, 1935). Moreover, the glycogen values reported in these studies have little or no significance, owing to the fact that very large errors in the determination of brain glycogen are unavoidable unless steps are taken to exclude or correct for the reducing substances other than glucose in the glycogen hydrolysate (Kerr, 1936).

In order to determine the nature of the lactic acid precursor we have compared the relationship of glycogen, free sugar, and lactic acid in resting brain with that in brain after various degrees of postmortem change.

### EXPERIMENTAL

*Effect of Anaerobic Autolysis*—In order to determine the final changes which occur anaerobically during autolysis of brain, the heads of five normal dogs decapitated under amytal anesthesia were incubated at 37° for about 2 hours. The brains were then removed, frozen in liquid air for the sake of accurate sampling, and specimens taken for glycogen, free fermentable sugar, and lactic acid. The analytical methods were those described in Paper II of this series (Kerr and Ghantus, 1936).

\* Holmes and Sherif extracted "soluble carbohydrate" and glycogen from brain with hot water. It has been shown (Pfüger, 1905) that it is impossible to extract glycogen completely with boiling water, owing possibly to combination of glycogen with other substances.

On comparison of the composition of incubated brain with that of brain frozen *in situ* in the living animal (Table I), it is seen that during incubation the free sugar disappears completely, and glycogen almost completely.

In the incubated brain of normal dogs the increase of lactic acid above the average for resting brain (131 to 175 mg., average 145) falls within the limits of that available from the glycogen and free sugar (131 to 230 mg., average 171). Since the average carbohydrate available as lactic acid precursor is not necessarily the same in the two series, the agreement is sufficiently close to justify the conclusion that both glycogen and free-sugar were converted to lactic acid.

We confirm the findings of Holmes and Sherif (1932) with respect to the dependence of the lactic acid maximum on the blood sugar level at death. The lactic acid formed during a 2 hour period of anaerobic incubation was determined on three dogs given 5 gm. of glucose per kilo of body weight intraperitoneally 1 hour before decapitation, and on three dogs in hypoglycemic shock as the result of injection of 15 units of insulin per kilo. The results (Table I) show that the amount of lactic acid formed on incubating brains from insulinized dogs cannot be accounted for on the assumption that its precursor is only the free sugar of brain, for the concentration of the latter is less in brain than in blood (Kerr and Ghantus, 1936). The total lactic acid formed is relatively small because both precursors (glycogen and free sugar) are sharply lowered by insulin (Kerr and Ghantus, 1936).

When the brains of hyperglycemic dogs were incubated, the lactic acid reached a level close to that of the blood sugar. Previous experience (Kerr and Ghantus, 1936) has shown that in hyperglycemia the free sugar of brain may be half to two-thirds that of blood, and this together with the normal content of glycogen (100 mg.) as precursor accounts for the lactic acid formed.

*Course of Glycolysis at Intervals*—In order to determine the relative rapidity of breakdown of free sugar and glycogen and of lactic acid formation, a series of analyses was made on brain fixed at intervals varying from 2 or 3 seconds to 30 minutes after excision. After the brain of a dog had been exposed under amytal anesthesia and the dura removed, two workers simultaneously excised the frontal lobes of the cerebrum with spoons and transferred each

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TABLE I

*Changes in Glycogen, Free Sugar, and Lactic Acid of Dog Brain during Anaerobic Incubation at 37°*

| Experiment No. | Remarks                                     | Blood sugar<br><br><i>mg. per 100 cc.</i> | Mg. per 100 gm. brain  |            |             |                    |
|----------------|---|---|------------------------|------------|-------------|--------------------|
|                |   |   | Glycogen (as glucose)* | Free sugar | Lactic acid | Total carbohydrate |
|                | Resting normal brain, frozen <i>in situ</i> |   |                        |            |             |                    |
|                | Minimum                                     |   | 86                     | 45         | 13          | 142†               |
|                | Maximum                                     |   | 144                    | 86         | 22          | 242†               |
|                | Average, 7 dogs†                            |   | 114                    | 57         | 18          | 189                |
| 133            | Normal, incubated 120 min.§                 |   | 3                      | 2          | 149         | 154                |
| 134            | “ “ 145 “                                   |   | 7                      | 0          | 193         | 200                |
| 135            | “ “ 145 “                                   |   | 3                      | 0          | 151         | 154                |
| 136            | “ “ 145 “                                   |   | 3                      | 0          | 160         | 163                |
| 137            | “ “ 145 “                                   |   | 2                      | 0          | 161         | 163                |
|                | Average                                     |   | 4                      | 0          | 163         | 167                |
| 174            | Hyperglycemia, incubated 120 min.           | 317                                       | 9                      |            | 330         |                    |
| 175            | “ “ 120 “                                   | 313                                       | 10                     |            | 304         |                    |
| 176            | “ “ 120 “                                   | 327                                       | 12                     |            | 318         |                    |
| 177            | Hypoglycemia (insulin), incubated 120 min.  | 20  | 7                      |            | 64          |                    |
| 178            | “ “ “                                       | 32  | 3                      |            | 98          |                    |
| 179            | “ “ “                                       | 29  | 0                      |            | 54          |                    |

\* Allowance has been made for the fact that 100 mg. of glycogen are equivalent to 111 mg. of glucose.

† The analyses for the individual animals are given in Table I of the paper by Kerr and Ghantus (1936).

‡ Minimum (or maximum) total carbohydrate of the seven animals, not the sum of the minima (or maxima).

§ The incubation period includes the interval between decapitation and freezing the brain in liquid air.

to a separate tared cylindrical specimen jar (provided with a cover and containing the fixing reagent<sup>4</sup>), and at once crushed the speci-

<sup>4</sup> The portion intended for glycogen determination was crushed in 5 cc. of 60 per cent KOH. After reweighing, the material was rinsed into the tube used for glycogen estimation with 10 cc. of alcohol. The specimen

mens with flat glass stoppers. The interval between cutting into the brain and the initial crushing was 2 to 3 seconds. The remainder of the two hemispheres was quickly transferred to a wooden block and divided into pieces of about 3 gm. each, and these were transferred in pairs to jars and crushed at definite time intervals.

Nine experiments in all were carried out with essentially the same results as those presented graphically in Figs. 1 and 2. Each figure represents two experiments, one covering the interval of 3 to 180 seconds, the other 3 to 30 minutes; hence the curves are not continuous. It is apparent that the free sugar is lost more rapidly than glycogen, the former disappearing completely in 3 to 5 minutes. The major part of the glycogen breakdown occurs in the first 15 minutes, the last 15 or 20 mg. disappearing more slowly during the next hour or two.

Within the first 3 minutes of postmortem change the curve of lactic acid production corresponds to the combined fall of free sugar and glycogen, whereas from that time on it is accounted for by the glycogen loss. While this indicates clearly the origin of lactic acid from both sources, it is not necessarily evidence for two processes for lactic acid production, a viewpoint maintained by Ashford and Holmes (1929) and Bumm and Fehrenbach (1931) among others.

Lactic acid at the 3 second interval is definitely above the resting level. It is a matter of some interest to know whether this is accounted for by a loss of glycogen or free sugar. In the experiments of Fig. 1 the lactic acid level at the 3 second interval is about 40 mg. above the resting average, and not more than 15 or 20 mg. of this could have arisen from free sugar, the resting level of which averages 57 mg. The entire lactic acid, however, could have originated from the glycogen, necessitating an initial concentration of about 120 mg. per 100 gm.

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for lactic acid and free sugar was likewise crushed in a jar containing 5 cc. of 1.2 N  $H_2SO_4$ , and reweighed; then 35 cc. of  $H_2O$  were added (the stopper being rinsed off). Next 5 cc. of 12 per cent  $ZnSO_4$  solution were added, stirred, and allowed to stand half an hour. The zinc was then precipitated by addition of 10 cc. of 0.8 N NaOH previously adjusted to the concentration necessary to bring the pH of the filtrate to about 8.0. This procedure is a modification of that of Friedemann and Graesser (1933), and the final concentrations of reagents are the same.

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Judging from this group of experiments as a whole (including five not presented for lack of space), it appears that the initial

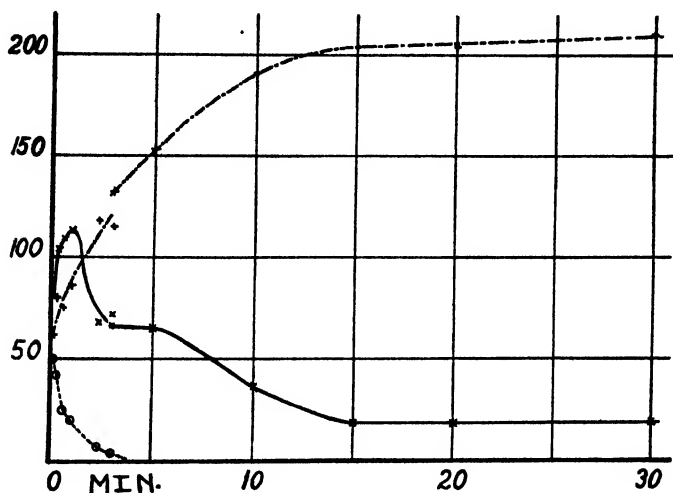


FIG. 1

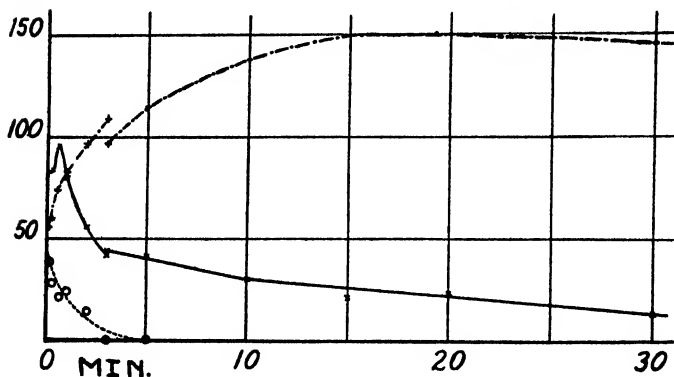


FIG. 2

FIGS. 1 AND 2. Changes in glycogen, free sugar, and lactic acid of dog brain during postmortem autolysis. The figures on the ordinate scale represent mg. per 100 gm. of brain. X, glycogen; O, free sugar; +, lactic acid.

rise of lactic acid may have its origin either from glycogen or free sugar or partly from each. The free sugar decreases relatively little, the average level at the 3 second interval in five

experiments being 51 mg., compared with an average of 57 mg. per cent for brain frozen *in situ*. The glycogen concentration 2 to 3 seconds after excision in six experiments varied from 69 to 85 mg. (average 77), whereas the resting values for seven dogs were within the range 77 to 130 mg. (average 102). The change represents an average decrease of 25 mg. per 100 gm. Since the glycogen content 3 seconds after death is still within the range of the normal resting brain, this average decrease cannot be taken as proof of change, but belief that such a change does occur is strengthened by the observation that the glycogen concentration after this interval is in no case equal even to the average glycogen content for normal resting brain.

The secondary rise in glycogen concentration observed in Figs. 1 and 2 (also found in four out of five other unpublished experiments) is an artifact, explained by a variation in the glycogen content of different parts of the brain. The glycogen increase is accompanied by a corresponding rise in total carbohydrate, hence does not represent a synthesis from free sugar. In these experiments the first pair of specimens crushed was the frontal lobes, followed by succeeding sections in order up to the posterior portions. That the second and third sections actually contain more glycogen than the others was shown in an experiment in which all sections were crushed at the same moment, the technique otherwise being identical with that in the experiments of Figs. 1 and 2. This conclusion has also been checked by analysis of different portions of brain frozen *in situ* with liquid air.

Loebel (1925) and Haarmann (1932) found that glycogen added to macerated brain was not converted to lactic acid. The fact that phosphocreatine disappears from brain within a few seconds after death (Kerr, 1935) suggests that labile constituents of the enzyme system governing glycolysis may also suffer postmortem change. Dickens and Greville (1933), in fact, showed that anaerobic substrate deprivation for 20 minutes resulted in a loss of the glycolytic power of brain. During this period adenosine-triphosphate decomposes to a large extent.<sup>5</sup> Hence we retested the ability of brain to convert glycogen (of liver) to lactic acid. Brain specimens were excised from anesthetized dogs and at once crushed in jars containing the glycogen in solution, while

<sup>5</sup> Unpublished experiments.



controls were crushed in saline. After 20 minutes both specimens were found to contain the same amount of lactic acid. During this period the major portion of the glycogen already present in brain is converted to lactic acid; hence the enzyme system is able to function. The inability to convert *added* glycogen to lactic acid would therefore appear to be due to some difference between the glycogen of brain and the purified liver glycogen added. We have prepared glycogen from mammalian brain and are unable to detect any differences in physical or chemical properties from that of liver.<sup>5</sup> It is suggested that glycogen under physiological conditions in tissues may be present in some form of combination more reactive than when uncombined. The presence of such compounds has been indicated by Willstätter and Margareth (1934) and by Pryzylecki, Giedroyc, and Rafalowska (1935). Experiments are in progress to test this hypothesis.

#### SUMMARY

During postmortem autolysis the free fermentable sugar of mammalian brain disappears within 3 to 5 minutes, and 80 to 85 per cent of the glycogen is lost within 15 minutes.

The amount of lactic acid formed during a 2 hour period of anaerobic incubation corresponds to the loss of glycogen and free sugar.

During the first 3 minutes of autolysis the lactic acid production corresponds to the combined fall of free sugar and glycogen, whereas from that time on it is accounted for by the glycogen loss alone.

The lactic acid maximum in autolyzed brain from hyperglycemic dogs is approximately equal to the blood sugar level at death, the precursors being the free sugar of brain (equal to half or two-thirds of the blood sugar level) and about 100 mg. of glycogen.

The lactic acid maximum in autolyzed brain from hypoglycemic (insulinized) dogs, although low, is about 3 times the blood sugar level, and again represents the amount of free sugar and glycogen available.

It is concluded that both glycogen and the free sugar of brain are precursors of lactic acid.

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# STUDIES ON THE INORGANIC COMPOSITION OF BLOOD

## IV. THE RELATIONSHIP OF POTASSIUM TO THE ACID-SOLUBLE PHOSPHORUS FRACTIONS

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Analyses made by Abderhalden (1898) of the blood of a number of mammals show that the erythrocytes of various species differ greatly in their content of potassium and sodium. His figures for cholesterol, proteins other than hemoglobin, and total phosphorus show similar differences, and these seem to be related to the variations in potassium and sodium, although in an irregular fashion.

We have extended the studies of Abderhalden for the purpose of learning whether the variations in potassium concentration might be correlated with those in one of the organic acid-soluble phosphorus compounds. Analyses of the erythrocytes of various vertebrates for total organic acid-soluble phosphorus, easily hydrolyzed organic phosphorus, and purine nucleotide were recently published (Kerr and Daoud, 1935). Below are presented the averages of determinations of potassium and sodium in the erythrocytes of about twenty species of vertebrates, together with the summarized results of the previous study of the distribution of phosphorus in the same species.<sup>1</sup> A comparison of these studies suggests a rough relationship between the potassium content and the organic acid-soluble phosphorus of erythrocytes, although these two constituents do not vary in a fixed ratio to each other.

<sup>1</sup> The greater part of the work reported here on potassium and sodium was done before Lohmann (1928) announced that tissues and blood contain "pyrophosphate." This accounts for the fact that certain bloods were examined for potassium, sodium, and organic phosphorus, but were not examined for their content of nucleotide and hydrolyzable phosphorus.

*Methods*

Blood, obtained by heart or vein puncture, or from the slaughter-house, was defibrinated by gentle stirring and centrifuged for 30 to 40 minutes at about 3500 R.P.M. After the serum and upper layer of leucocytes were removed as completely as possible, the remaining unwashed erythrocytes<sup>2</sup> were weighed in volumetric flasks and laked with water. Trichloroacetic acid was added to make a final concentration of 8 per cent, and after dilution to definite volume the mixture was filtered. The usual dilution was 1:10. The combined blood of several individuals was analyzed when small animals were studied (chicken, partridge, guinea pig, rat).

Potassium was determined by the method of Shohl and Bennett (1928). For the determination of sodium, aliquot portions of the filtrate were ashed exactly as in the potassium method, and the procedure of Kramer and Gittleman (1924-25) applied to the dissolved ash. All determinations were performed in duplicate except in the few cases in which the material available was too small.

## DISCUSSION

In order to present all results in terms of the same unit, the data from the previous publication on the distribution of phosphorus in erythrocytes (Kerr and Daoud, 1935) have been converted into mm per 1000 gm. of cells; the figure 1.088<sup>3</sup> was arbitrarily used for the specific gravity of all species. In Table I the average contents of potassium, sodium, organic acid-soluble phosphorus, nucleotide, hydrolyzable phosphorus, and the residual undetermined phosphorus are compared for the various species examined. The relationship of potassium to the organic phosphorus fractions is represented graphically in Fig. 1, in which the sum of the hydrolyzable phosphorus and nucleotide has been represented as a single substance inasmuch as evidence presented in the earlier publication indicates that these two fractions are combined as adenosinetriphosphate. Although there is no constancy about

<sup>2</sup> It has been shown (Kerr, 1929-30) that washing erythrocytes in isotonic saline solutions leads to changes in the concentration of potassium and sodium within the cell.

<sup>3</sup> Based on data of Abderhalden, cited in Mathews (1925).

TABLE I

Comparison of Average Molar Concentration of Potassium, Sodium, Total Organic Acid-Soluble Phosphorus, Hydrolyzable Phosphorus, Nucleotide and Undetermined Phosphorus in Erythrocytes of Various Vertebrates

| Species   | No. of specimens | mm per 1000 gm. corpuscles |        |                    | mm per 1000 gm.*             |                                |              |                |
|---|------------------|----------------------------|--------|--------------------|------------------------------|--------------------------------|--------------|----------------|
|   |                  | Potassium                  | Sodium | Potassium + sodium | Total organic acid-soluble P | Organic P hydrolysed in 7 min. | Nucleotide P | Undetermined P |
| Mammals   |                  |                            |        |                    |                              |                                |              |                |
| Carnivores  |                  |                            |        |                    |                              |                                |              |                |
| Dog ( <i>Canis familiaris</i> ).....                  | 28               | 8.7                        | 107.0  | 115.7              | 16.8                         | 2.1                            | 1.1          | 13.6           |
| Cat ( <i>Felis domestica</i> ).....                   | 4                | 5.9                        | 103.7  | 109.6              | 6.2                          | 1.9                            | 0.9          | 3.4            |
| Rodents   |                  |                            |        |                    |                              |                                |              |                |
| Rabbit ( <i>Lepus cuniculus</i> )....                 | 3                | 99.1                       | 16.0   | 115.1              | 26.1                         | 4.8                            | 2.3          | 19.0           |
| Mole-rat ( <i>Spalax</i> ).....                       | 1                | 104.5                      |        |                    | 17.6                         |                                |              |                |
| Albino rat ( <i>Mus norvegicus albinus</i> ).....     | 1                | 100.5                      | 11.9   | 112.4              | 14.9                         | 2.3                            | 1.2          | 11.4           |
| Guinea pig ( <i>Cavia domestica</i> )..               | 5                | 104.5                      | 15.0   | 119.5              | 19.2                         | 2.7                            | 1.7          | 14.8           |
| Primates  |                  |                            |        |                    |                              |                                |              |                |
| Man.....  | 15               | 109.5†                     |        |                    | 14.5                         | 3.6                            | 1.9          | 9.0            |
| Monkey ( <i>Macacus</i> ).....                        | 1                | 111.5                      |        |                    | 13.2                         | 2.6                            | 1.1          | 9.5            |
| Ungulates   |                  |                            |        |                    |                              |                                |              |                |
| Pig ( <i>Sus scrofa, melitensis</i> )...              | 4                | 99.5                       | 10.8   | 110.3              | 28.1                         | 6.8                            | 3.2          | 18.1           |
| Ox ( <i>Bos taurus</i> ).....                         | 6                | 21.8                       | 79.0   | 100.8              | 2.7                          | 1.0                            | 0.6          | 1.1            |
| Goat ( <i>Capra hircus, syriaca</i> )..               | 6                | 18.4                       | 93.2   | 111.6              | 3.6                          | 1.7                            | 1.0          | 0.9            |
| Gazelle ( <i>Gazella dorcas</i> ).....                | 1                | 37.5                       | 75.2   | 112.7              | 2.0                          |                                |              |                |
| Sheep ( <i>Ovis aries, crassican-</i><br><i>dus</i> ) |                  |                            |        |                    |                              |                                |              |                |
| Group I.....  | 4                | 18.4                       | 83.5   | 101.9              | 4.2                          | 1.8                            | 1.1          | 1.3            |
| “ II.....   | 11               | 64.2                       | 15.6   | 79.8               |                              |                                |              |                |
| “ III.....  | 2                | 58.1                       | 46.0   | 104.1              |                              |                                |              |                |
| Camel ( <i>Camelus dromedarius</i> )                  | 5                | 50.1                       | 19.6   | 69.7               | 18.7                         | 2.7                            | 1.7          | 14.3           |
| Horse ( <i>Equus caballus</i> ).....                  | 2                | 87.7‡                      |        |                    | 13.9                         | 0.9                            | 0.5          | 12.5           |
| Mule.....   | 1                | 92.1                       |        |                    | 13.5                         | 1.3                            | 0.6          | 11.6           |

\* Calculated from data of Kerr and Daoud (1935), assuming a specific gravity of 1.088 for cells of all species.

† Average of analyses by Kramer and Tisdall (1922).

‡ Calculated from data of Abderhalden (1898).

TABLE I—Concluded

| Species   | No. of specimens | mm per 1000 gm. corpuscles |        |                    | mm per 1000 gm.*             |                                |              |                |
|---|------------------|----------------------------|--------|--------------------|------------------------------|--------------------------------|--------------|----------------|
|   |                  | Potassium                  | Sodium | Potassium + sodium | Total organic acid-soluble P | Organic P hydrolysed in 7 min. | Nucleotide P | Undetermined P |
| Birds   |                  |                            |        |                    |                              |                                |              |                |
| Fowl  |                  |                            |        |                    |                              |                                |              |                |
| Chicken ( <i>Gallus bankiva</i> ). . .                                | 4                | 97.3                       | 7.1    | 104.4              | 29.3                         | 2.9                            | 1.7          | 24.7           |
| Turkey ( <i>Meleagris gallopavo</i> ). .                              | 4                | 99.5                       | 9.7    | 109.2              | 27.8                         | 5.2                            | 3.0          | 19.6           |
| Guinea hen ( <i>Numida meleagris</i> ) . . . . .                      | 3                | 105.6                      | 10.3   | 115.9              | 30.2                         |                                |              |                |
| Partridge ( <i>Caccabis saxatilis</i> ,<br><i>syriaca</i> ) . . . . . | 1                | 128.7                      |        |                    | 33.0                         |                                |              |                |
| Goose ( <i>Anser domesticus</i> ) . . .                               | 4                | 89.9                       | 6.8    | 96.7               | 30.0                         | 6.3                            | 3.0          | 20.1           |

the molar ratio of potassium to any of the phosphorus fractions, in all those bloods with high organic acid-soluble phosphorus (13.2 mm per 1000 gm. or over) potassium is likewise high in concentration, with the single exception of the dog. Moreover, all bloods with low potassium content (37.5 mm or less per 1000 gm. of corpuscles) have only a small amount of organic phosphorus (6.2 mm or less), with the exception of the dog. The molar ratio of potassium to the organic phosphorus varies from 0.6:1 in dog corpuscles to a maximum of 19:1 in those of the gazelle. The cells of the dog are the only ones examined in which the molar concentration of potassium is less than that of organic phosphorus. In cat corpuscles the two are approximately equal.

Since the hydrolyzable phosphorus and nucleotide phosphorus comprise only a small fraction of the organic acid-soluble phosphorus in most species, except those with low phosphorus content, what has been said of the relationship of potassium to the total organic phosphorus applies for the most part also to the undetermined fraction.

In spite of the fact that in general low potassium is associated with low phosphorus, and high potassium with high phosphorus, these two constituents vary greatly without exhibiting any con-

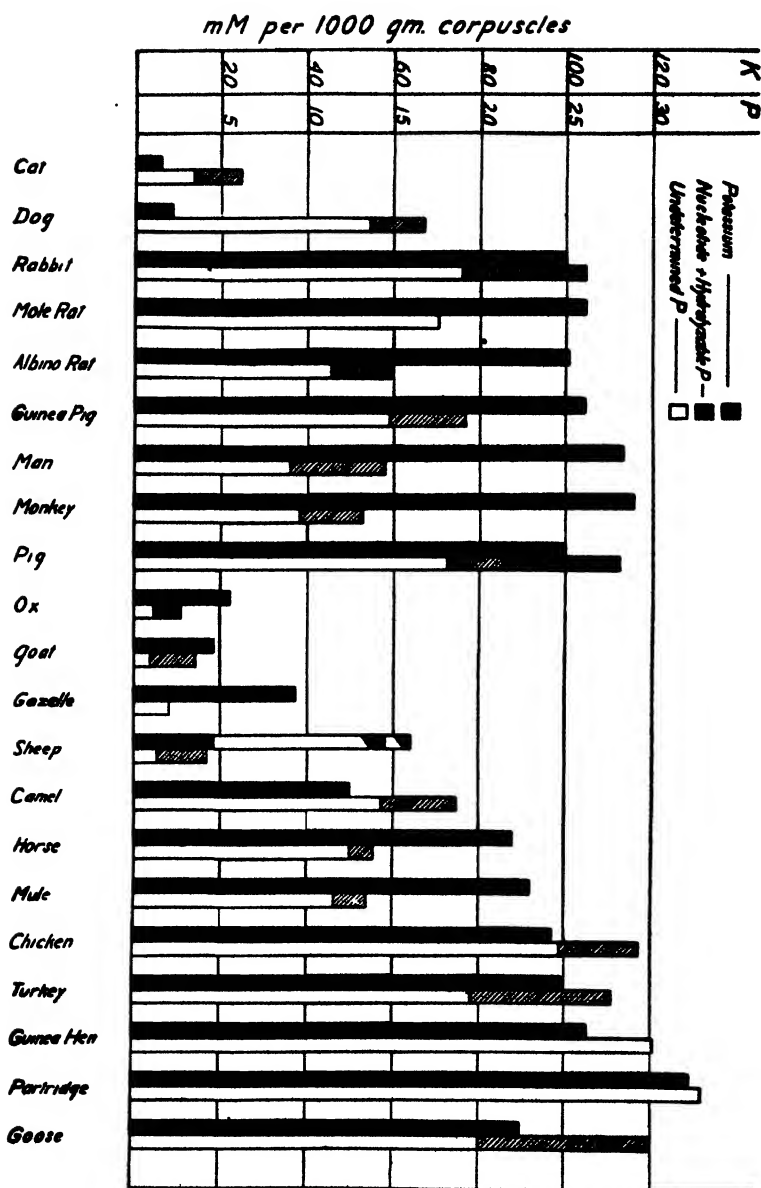


FIG. 1. Relation of potassium to organic phosphorus fractions in erythrocytes of various animals.



sistent relationship to each other. This is best illustrated in the case of sheep, where large differences in the potassium content of the erythrocytes are found in different races, whereas the organic phosphorus varies within narrow limits. Likewise large variations in potassium occur between the dog, guinea pig, and camel, all of which have approximately the same amount of organic phosphorus.

There is considerable variation in blood composition among different species of the same order. The camel and pig both differ strikingly from the other ungulates in respect to both the potassium and all the phosphorus fractions of their erythrocytes. Judging from blood composition, therefore, neither of these animals seems to belong in the same group with the ox, goat, sheep, and gazelle. Pig corpuscles resemble those of the rabbit closely, while the camel seems to be in a class by itself.

Occasional differences between individuals of the same species are observed, the potassium concentration in the erythrocytes of dogs varying between 6.4 and 16.3 mm per 1000 gm. Changes in the potassium content of dog erythrocytes under certain experimental conditions have been reported previously (Kerr, 1926).

Perhaps the most surprising feature revealed by our analyses is the wide variation in the blood composition of various sheep. Although individuals of the same species as a rule have similar chemical composition, with minor fluctuations, there are enormous differences among individual sheep in their content of potassium, while the total organic phosphorus remains nearly constant. The results of the individual analyses of sheep corpuscles are presented in Table II. The sheep have been grouped arbitrarily into three classes on the basis of the potassium and sodium content of their erythrocytes. Our analyses for Group I (low potassium, high sodium) are similar to the data given by Abderhalden (1898) for sheep blood. In Group II, however, we appear to have a different race, with high potassium and low sodium concentration. In Group III the potassium content is similar to that of Group II, but sodium is considerably higher. All the blood specimens were from the oriental fat-tailed sheep, a group which includes the mixed descendants of several races, modified by selection on the part of the Arabs to produce enormous tails.

Aside from the fact that the erythrocytes of the only two car-

nivorous mammals studied contain little potassium (in spite of the predominance of potassium over sodium in flesh) there seems to be no relationship between diet and the potassium content of the blood corpuscles. The herbivorous ungulates differ greatly

TABLE II

*Potassium, Sodium, and Organic Phosphorus in Unwashed Erythrocytes of Sheep (Ovis aries, crassicaudus)*

| Group No.    | Mg. per 100 gm. corpuscles |             |                        |     |     |
|--------------|----------------------------|-------------|------------------------|-----|-----|
|              | Total acid-soluble P       | Inorganic P | Organic acid-soluble P | K   | Na  |
| I            | 13.1                       | 2.2         | 10.9                   | 38  | 164 |
|              | 9.1                        |             |                        | 72  | 226 |
|              | 12.0                       |             |                        | 82  | 193 |
|              | 13.4                       |             |                        | 95  | 183 |
| Average..... | 11.9                       | 2.2         | 9.7                    | 72  | 192 |
| II           | 14.8                       | 2.5         | 12.3                   | 194 |     |
|              | 15.8                       | 4.2         | 11.6                   | 207 | 23  |
|              | 13.1                       | 2.0         | 11.1                   | 209 | 20  |
|              | 13.1                       | 2.2         | 10.9                   | 211 | 41  |
|              | 14.6                       |             |                        | 288 | 48  |
|              | 14.5                       |             |                        | 289 | 69  |
|              | 12.7                       | 1.9         | 10.8                   | 337 | 38  |
|              |                            |             |                        | 210 | 36  |
|              |                            |             |                        | 291 | 39  |
|              |                            |             |                        | 208 | 16  |
|              |                            |             |                        | 319 | 33  |
| Average..... | 14.1                       | 2.6         | 11.5                   | 251 | 36  |
| III          | 13.2                       |             |                        | 223 | 110 |
|              | 12.1                       | 2.5         | 9.6                    | 231 | 102 |
| Average..... | 12.7                       | 2.5         | 10.2                   | 227 | 106 |

from each other, the corpuscles of pig and horse containing practically no sodium, the others both potassium and sodium. The large difference in the potassium content of the cells of different groups of sheep, all of which have the same diet, illustrates how little the diet influences the mineral content of the erythrocytes.

The finding of sodium in the erythrocytes of species having high potassium concentration demands explanation, inasmuch as Abderhalden (1898) found no sodium in the erythrocytes of the horse, pig, and rabbit. In our experiments the corpuscles were separated by centrifugation and not washed.<sup>2</sup> The small amount of sodium found in the corpuscles of high potassium content may therefore be due to the presence of serum incompletely separated during centrifugation.<sup>4</sup>

Even after allowance is made for error due to admixed serum or adsorbed sodium, the sum of the molar concentrations of potassium and sodium is not uniform. The most marked variations from the average occur in the blood of camels and certain sheep, in which there is little sodium and the sum of sodium and potassium is about two-thirds of that found in most animals. The amount of base seems to be unrelated to the quantity of acid-soluble phosphorus present. Further study is necessary to determine whether or not the low concentration of base is associated with a low protein content. Abderhalden (1898) found great variation in the protein (other than hemoglobin) of the corpuscles of different species.

Although the *concentration* of the mineral elements in plasma may vary in different vertebrates, the *proportion* which these elements bear to each other is practically uniform. This uniformity in the mineral content of vertebrate plasma is an endowment, as Macallum (1926) has shown, from the early zoological period when a closed circulatory system first developed, and has been maintained by the activity of the kidneys. No explanation is as yet available for the fact that the erythrocytes of various vertebrates, though bathed in a fluid identical with respect to the proportion of its mineral elements, differ markedly in their content of potassium and sodium. As already stated, cholesterol and protein (other than hemoglobin) are also variable in the corpuscles of different species, and it may be that the varying potassium content is correlated with one of these in addition to a partial dependence upon organic phosphorus compounds.

<sup>4</sup>Oberst (1934) found that human red blood cells washed with isotonic Na-free serum still contain 7 mm of Na per 1000 gm. as compared with 12 mm for unwashed cells.

## SUMMARY

1. The potassium and sodium content of the erythrocytes of twenty species of vertebrates has been determined and compared with the concentration of various fractions of the organic acid-soluble phosphorus.

2. A low concentration of potassium (37.5 mm per 1000 gm. and less) is associated with low organic phosphorus content (6.2 mm or less) in all species examined except the dog. A high level of organic acid-soluble phosphorus (13.2 mm or more per 1000 gm.) is likewise associated with high levels of potassium, the dog again being an exception.

3. Great differences are found in the composition of the erythrocytes of various species of the same order, and in different races of the same species. The concentration of potassium and sodium varies greatly in the blood of different sheep.

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## THE OXIDATION OF ASCORBIC ACID AND ITS REDUCTION IN VITRO AND IN VIVO\*

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The outstanding chemical property of ascorbic acid (vitamin C) is that it is a reducing agent. The suggestion is obvious that its physiological function may be associated with this property, and, if it is oxidized reversibly, with its behavior in an oxidation-reduction system. It is desirable therefore to know the oxidation-reduction potential of ascorbic acid.

A number of attempts were made to measure this potential (2-6) both before and after it was established that ascorbic acid (earlier called hexuronic acid) and vitamin C were identical. In all except one of these studies it was reported that ascorbic acid does not yield thermodynamically reversible potentials. More or less rapid negative potential drifts were observed and the final steady value was independent of the initial concentration of the oxidized form. The one claim that a reversible potential was obtained (3) was based upon inadequate evidence; and later studies have shown that the order of magnitude of the potential given was widely inaccurate.

The difficulty here is 2-fold. Both the reduced and the reversibly oxidized forms of ascorbic acid react slowly with the electrode, and, what is more important, the reversibly oxidized

\* Nearly all of the work described in this paper, with the exception of the glutathione experiments, was presented at the meeting of the American Chemical Society at San Francisco, August, 1935. Its submission for publication was delayed for the sake of presenting a more complete treatment of the reducing mechanism of oxidized ascorbic acid in the tissues. A preliminary account of some of the glutathione experiments has appeared (1).

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form, now commonly called dehydroascorbic acid, undergoes an irreversible change in aqueous solution above pH 4 at ordinary temperatures. When these two factors were taken into account, it was possible to obtain a fairly accurate value for the reversible potential of the first oxidation stage (7). This behavior of ascorbic acid and the value of the potential given were confirmed by Wurmser and de Loureiro (8). Somewhat later Fruton (9) reported values for this potential 100 millivolts more negative than those obtained by Wurmser and de Loureiro and by ourselves. It is shown below that the potential reported by Fruton pertains to a second oxidation stage.

This instability of dehydroascorbic acid is responsible for the variation in antiscorbutic potency of oxidized solutions of the vitamin, as well as for the complex behavior of ascorbic acid *in vitro*. The greater part of the present study deals with different aspects of this irreversible change. We have attempted on the one hand to elucidate some of the difficulties which have been encountered in determining the reversible oxidation-reduction potential of ascorbic acid, and on the other to follow, guided by the *in vitro* findings, the fate of dehydroascorbic acid *in vivo*.

The work reported here falls into four parts—physicochemical measurements, nutrition, and physiological experiments, and a study of the interaction of oxidized ascorbic acid and glutathione. In order to facilitate following the argument through a varied and extended series of experiments we shall present here a description of the main features of the irreversible change in dehydroascorbic acid. As stated, dehydroascorbic acid undergoes a spontaneous, irreversible change at ordinary temperatures in aqueous solution at hydrogen ion concentrations less than pH 4. This change is responsible for the negative potential drift observed in electro-metric measurements of the oxidation-reduction potential, and for the loss in reversibility of the first oxidation stage. The product of this change is a stronger acid than dehydroascorbic acid, and is a more powerful reducing agent than ascorbic acid itself. It is distinguished from dehydroascorbic acid also in that it is not reduced by  $H_2S$  in acid solution, nor by glutathione in neutral or alkaline solution. It is not antiscorbutic; whereas dehydroascorbic acid possesses very nearly the same antiscorbutic potency as the reduced form of the vitamin (the form in which most of it is

found in nature), although the "half-life" of dehydroascorbic acid *in vitro* at the pH and temperature of the tissues is only a few minutes. The rates of appearance of all of these manifestations of the irreversible change in dehydroascorbic acid exhibit the same dependence on the hydrogen ion concentration. They are also all independent of the presence of air or oxidizing agents. The irreversible change is therefore not an oxidation. It is also independent of the oxidizing agent used to form dehydroascorbic acid. The resolution of the paradox between the high antiscorbutic potency of dehydroascorbic acid and its rapid loss in potency *in vitro* at the pH and temperature of the blood and tissues turned out to be simply that dehydroascorbic acid is rapidly reduced *in vivo* to ascorbic acid. The principal reducing agent here is glutathione. Apart from this rapid reduction we found no evidence of any greater stability of dehydroascorbic acid *per se* in blood or in the tissues than *in vitro*.

#### *Physicochemical Measurements*

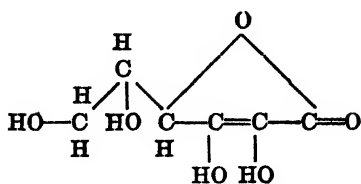
An account of these measurements is presented first because these data are necessary for an appraisal of the possibilities of ascorbic acid as a reducing agent *in vivo*.

Ascorbic acid can be made to take up the equivalent of at least 3 atoms of oxygen in the course of its oxidation, in three separate steps. The oxidation-reduction potentials of these three steps were estimated, the potential of the first by electrometric and colorimetric methods, of the second and third by a colorimetric method only, and with less precision.

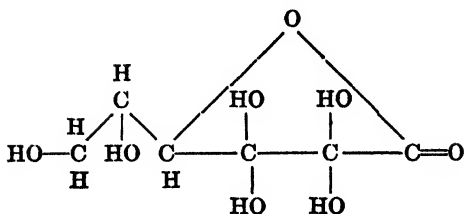
We also determined the orders of magnitude of the first acid dissociation constants of dehydroascorbic acid and of the product of its irreversible change.

Interposed between the three oxidation stages are several irreversible non-oxidative changes. In order to make the description of the interrelation of all these reactions clearer we shall present here the formulæ for the different compounds involved and the relations which we propose exist between them. The formulæ are those given by Herbert *et al.* (10). Ascorbic acid is represented by formula (I); dehydroascorbic acid by (II). It is the hydrated oxidation product of (I). The first reversible oxidation stage is

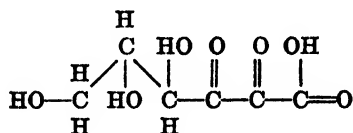




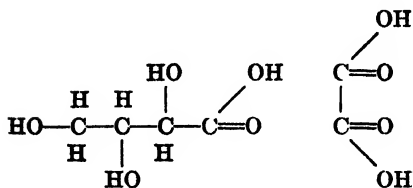
I



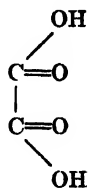
II



III



IV



V

(I)  $\rightleftharpoons$  (II). (III) is the product of the irreversible change in dehydroascorbic acid. From formula (III) it is 2,3-diketo-*L*-gulonic acid. We shall refer to it hereafter as diketogulonic acid. In the second oxidation diketogulonic acid eventually gives rise to *L*-threonic acid (IV) and oxalic acid (V). It is at present uncertain whether the reversible step in the second oxidation stage is (III)  $\rightleftharpoons$  (IV) + (V), or from (III) to some intermediary compound which eventually breaks down to (IV) and (V). It would seem

that the second alternative is the more probable. In the third oxidation stage we are uncertain even regarding the compound which is oxidized. It is a rapid reaction only on the alkaline side of neutrality. Tentatively we would suggest that it is the *l*-threonic acid (IV) which is oxidized, and that the alkaline reaction is necessary for the cleavage of the intermediary which is in equilibrium with diketogulonic acid in the reversible second oxidation stage.

We wish to emphasize that neither the magnitude of the oxidation-reduction potentials and ionization constants given, nor the properties of the reactions described below, nor the application of this information to physiological questions depends in any way on structural considerations. Wherever reference is made below to diketogulonic acid, what is meant is the product of the irreversible non-oxidative change in dehydroascorbic acid.

*Electrometric Measurement of Oxidation-Reduction Potential of First Oxidation Stage*—These measurements were carried out at 35.5° with the vacuum technique previously described (11). The principle of the method is the measurement of the potentials acquired by noble metal electrodes when these are immersed in solutions containing known proportions of the reduced and oxidized forms of the substance investigated.

Mixtures containing varying proportions of reduced and oxidized ascorbic acid were made in citrate-phosphate buffers at the hydrogen ion concentrations given in Table I. The oxidized form was prepared by oxidation of ascorbic acid with iodine. The specimen of ascorbic acid used gave a titration value of 100 per cent on the basis that 1 molecule of ascorbic acid requires 2 equivalents of iodine. No reversibly oxidizable dye nor enzyme was added. All solutions used were kept at ice temperature and rendered nearly air-free by aeration with nitrogen. The different mixtures were prepared immediately before their transfer to the electrode vessels. These were evacuated with an oil pump for 3 minutes at room temperature, followed by 2 minutes at 35°. During the evacuation at room temperature the solutions froze. Platinum foil electrodes were used. The same results were obtained with plain or gold-plated electrodes. The electrode vessels were rocked continuously in an air bath maintained at 35.5°. Readings were taken at frequent intervals until either a steady

potential was established or a uniform drift of the potential was observed.

TABLE I

*Oxidation-Reduction Potentials at 35.5° of Different Mixtures of Reduced and Oxidized Ascorbic Acid at Different Hydrogen Ion Concentrations*

| pH   | Concentration of reduced form | Concentration of oxidized form | Reduced<br>Oxidized | Calculated potential difference = 0.0306 log (reduced)/(oxidized) | Observed potential difference* | E's values; i.e., when (reduced) + (oxidized) = 1 |          | Potential drift -dE/dt | Period of observation |                    |
|------|-------------------------------|--------------------------------|---------------------|---|--------------------------------|---|----------|------------------------|-----------------------|--------------------|
|      |                               |                                |                     |   |                                | Calculated  | Observed |                        | Total                 | Of constant -dE/dt |
|      | moles × 10 <sup>4</sup>       | moles × 10 <sup>4</sup>        |                     | mv.   | mv.                            | mv.   | mv.      | mv. per hr.            | min.                  | min.               |
| 2.04 | 2.43                          | 0.05                           | 4.82                | 18  | 17                             | +0.281  | +0.283   | 0                      | 465                   | 75                 |
| 2.04 | 2.43                          | 2.12                           | 1.20                |   |                                | +0.281  | +0.281   | 0                      | 420                   | 75                 |
| 2.68 | 0.74                          | 1.38                           | 0.54                | 18  | 18                             | +0.242  | +0.242   | 0                      | 705                   | 75                 |
| 2.68 | 2.96                          | 1.38                           | 2.14                |   |                                | +0.242  | +0.242   | 0                      | 700                   | 95                 |
| 3.30 | 0.72                          | 1.41                           | 0.51                | 18  | 17                             | +0.204  | +0.205   | 0                      | 240                   | 80                 |
| 3.30 | 2.87                          | 1.41                           | 2.03                |   |                                | +0.204  | +0.204   | 0                      | 235                   | 75                 |
| 4.01 | 0.73                          | 1.40                           | 0.52                | 18  | 19                             | +0.166  | +0.167   | 1.4                    | 265                   | 150                |
| 4.01 | 2.93                          | 1.40                           | 2.07                |   |                                | +0.166  | +0.166   | 1.4                    | 260                   | 165                |
| 4.65 | 0.75                          | 1.38                           | 0.55                | 18  | 19                             | +0.138  | +0.137   | 1.9                    | 370                   | 120                |
| 4.65 | 3.01                          | 1.38                           | 2.18                |   |                                | +0.138  | +0.136   | 1.6                    | 365                   | 120                |
| 5.31 | 1.91                          | 1.82                           | 1.05                | 18  | 17                             | +0.119  | +0.118   | 3.0                    | 580                   | 270                |
| 5.31 | 1.91                          | 0.45                           | 4.20                |   |                                | +0.119  | +0.120   | 3.0                    | 570                   | 240                |
| 5.75 | 4.55                          | 0.56                           | 8.18                | 37  | 38                             | +0.106  | +0.108   | 5.9                    | 440                   | 210                |
| 5.75 | 1.14                          | 2.23                           | 0.51                |   |                                | +0.106  | +0.109   | 7.2                    | 435                   | 240                |
| 6.43 | 3.81                          | 1.52                           | 2.50                | 18  | -49                            | +0.080  | +0.096   | 20.9                   | 295                   | 110                |
| 6.43 | 0.95                          | 1.52                           | 0.63                |   |                                | +0.080  | +0.045   | 31.3                   | 290                   | 110                |

\* The minus sign of the observed potential difference for the pair at pH 6.43 indicates that the mixture with the lower ratio of reduced to oxidized ascorbic acid was 31 millivolts more negative instead of 18 millivolts more positive than the other member of this pair.

The electrode equation was obtained by the derivation described earlier (11). For the mechanism, oxidized ascorbic acid + 2H<sup>+</sup> + 2(e) → reduced ascorbic acid, the equation is

$$E_{\text{obs.}} = E - \frac{RT}{F} \text{pH} - \frac{RT}{2F} \ln \frac{(\text{reduced})}{(\text{oxidized})} - \frac{RT}{2F} \ln \frac{K_r}{K_o} \\ - \frac{RT}{2F} \ln \frac{K_o + (\text{H}^+)}{K_r + (\text{H}^+)}$$

where (reduced) and (oxidized) indicate the concentrations of the total reduced and oxidized forms respectively,  $K_r$  and  $K_o$  the first dissociation constants of the reduced and oxidized forms considered as monovalent acids. For simplicity we have ignored the second dissociation constants. Birch and Harris (12), and Karrer *et al.* (4), found the value of  $\text{p}K_r$  to be about 4.17. By a colorimetric titration procedure described below we found the value of  $\text{p}K_o$  to be approximately 9.0. The value of  $n$  was taken as 2, which was indicated by the iodine titration and corroborated by the agreement between the observed and calculated  $E'_0$  values in Table I.

The data in Table I may be divided into two groups: the three most acid pairs in which the potentials attained steady values, and the remaining five from pH 4.01 to 6.43 inclusive in which the potentials did not attain steady values. In the latter group, after the first rapid negative change, the potential changes slowed down to drifts which were uniform for hours; *i.e.*,  $-dE/dt$  in each case was a constant. The magnitude of this constant was greater the higher the pH.

The notable feature of the first group of data is that several hours elapsed before steady potentials were attained. Once attained the differences between these values in the different mixtures were sufficiently close to the theoretical differences for the differences in the ratios of (reduced)/(oxidized) to warrant the conclusion that they are thermodynamically reversible potentials. This conclusion is supported further by the fact that the  $E'_0$  values ( $E'_0$  is the calculated potential at any specified pH when (reduced)/(oxidized) = 1) fell on the theoretical curve calculated from the electrode equation. The values in this pH region, from 2 to 3.3 inclusive, have been confirmed by Wurmser and de Loureiro who employed a similar electrometric method (8), by our later colorimetric measurements, and by Ball (13) who has worked out a rapid electrometric method (Table IV).

The second group of data in Table I does not give values of the

thermodynamically reversible potentials directly. Such values were obtained on the assumption first, that the constant negative drifts which characterize this group are the result of an irreversible change in the oxidized form (dehydroascorbic acid) described above, and second, that this irreversible change is a first order reaction. On the basis of the second assumption it follows that  $d \log (\text{oxidized})/dt$  is a constant. From the electrode equation it is seen that the potential,  $E_{\text{obs.}}$ , is a linear function of  $\log (\text{oxidized})$ .  $-dE_{\text{obs.}}/dt$  will therefore be a constant. This was observed in every case. The  $E'_0$  values given in Table I were obtained on the basis of these two assumptions by subtracting from the observed potential at any moment during the interval in which  $-dE_{\text{obs.}}/dt$  was constant the product of the value of this constant and the time which had elapsed from the moment when the mixture containing the oxidized ascorbic acid was brought to  $35^\circ$ ; i.e.,  $(-dE_{\text{obs.}}/dt) \times t$ .

The  $E'_0$  values so obtained fell on the same theoretical  $E'_0$ -pH curve as those in the more acid group which were obtained without these assumptions or extrapolations. The theoretical  $E'_0$ -pH curve for ascorbic acid changes its slope at pH 4.2 from 60 to 30 millivolts per pH unit. The extrapolated  $E'_0$  values fell on this curve both in the region in which the slope is changing and on the later straight line portion. This coincidence is the more striking because the extrapolation slopes are progressively steeper with increasing pH. The values given in the recent paper by Ball (Table IV) are in accord with the order of magnitude of the  $E'_0$  values obtained by our extrapolation.

At pH 6.43 the situation became too complicated to be interpreted. The potential difference between the two mixtures was in the reverse of the theoretical direction; the extrapolation curves were very steep and different in the two mixtures. As a result the extrapolated  $E'_0$  values were different for the two mixtures, and both were more positive than the theoretical curve at this pH. We have set these values aside for these reasons, and also because diketogulonic acid begins to behave as a reducing agent at this pH (see below) and presumably therefore also affects the electrode in the manner of a reductant. This introduces a number of disturbing effects on the electrode potential which we cannot discuss here. Their influence is small up to pH 5.75. Beyond this point they dominate the picture.

*Relation between Regeneration of Ascorbic Acid by  $H_2S$  in Solutions of Dehydroascorbic Acid and Rate of Irreversible Change in Dehydroascorbic Acid*—In this and in the next two sections we shall present independent evidence that the negative drift in the electrode potentials observed at hydrogen ion concentrations less than at pH 4 is a consequence, as we have assumed above, of a spontaneous irreversible change in dehydroascorbic acid.

Dehydroascorbic acid is restored practically quantitatively to ascorbic acid by  $H_2S$  in acid solution. After its conversion to diketogulonic acid this property is lost. Table II is a summary of some experiments which show that the irreversible change in dehydroascorbic acid, judged by this criterion, begins at about pH

TABLE II

*Variation with pH of Rate of Irreversible Change in Dehydroascorbic Acid Measured by Yield of Ascorbic Acid Recovered after Treatment with  $H_2S$*

| pH  | Per cent of original reducing capacity recovered by treatment with $H_2S$ after incubation in vacuo at 23° for |        |        |        |
|-----|--|--------|--------|--------|
|     | 1 hr.  | 2 hrs. | 4 hrs. | 6 hrs. |
| 3.0 | 100  | 95     | 98     | 98     |
| 4.0 | 98   | 98     | 100    | 98     |
| 4.5 | 95   | 93     | 90     | 88     |
| 6.0 | 86   | 80     | 73     | 60     |
| 7.0 | 61   | 41     | 30     | 20     |
| 8.0 | 21   | 18     | 15     | 14     |
| 9.0 | 16   | 12     | 11     | 11     |

4, and becomes progressively faster with increasing pH above this point. The variation with pH in the behavior toward  $H_2S$  is the same in both these respects as the electrode potential drifts. The failure of  $H_2S$  to regenerate ascorbic acid from diketogulonic acid shows that dehydroascorbic acid and diketogulonic acid are not in equilibrium, and that the change is therefore an irreversible one.

The experimental procedure was as follows: the buffer solution used (McIlvaine's series) was pipetted into the main, lower compartment of a Thunberg tube, and the plain aqueous ascorbic acid solution previously oxidized with iodine, into the overhang. After evacuation with an oil pump the contents of the tube were mixed and then allowed to stand at room temperature (20–25°) for the times indicated. At the end of the specified period the

vacuum was broken, 0.1 N hydrochloric acid was immediately added to bring the pH to 2.0, the contents of the tube were transferred to a small Erlenmeyer flask, hydrogen sulfide was bubbled through for 3 hours, after which the 2-way stop-cock was closed and the solution allowed to stand overnight under hydrogen sulfide. Next morning this was removed by a stream of nitrogen, and the solutions titrated with 2,6-dichlorophenol indophenol.

Barron and his collaborators (14) measured the rates of irreversible oxidation of ascorbic acid by oxygen (catalyzed by  $\text{CuCl}_2$ ) at different hydrogen ion concentrations. Their figures are essentially the same as those in Table II, which represent the rates of irreversible change in dehydroascorbic acid *in vacuo* (and in the absence of oxidizing agents). It is therefore probable that the irreversibility in the "irreversible" oxidation of ascorbic acid at hydrogen ion concentrations less than at pH 4 resides in the non-oxidative change of dehydroascorbic to diketogulonic acid, and not in any special mode of oxidation of ascorbic acid prior to this change. Accordingly the rate of its "irreversible" oxidation is governed by the rate of this irreversible change.

*Inability of Glutathione to Regenerate Ascorbic Acid from Products of Irreversible Change in Dehydroascorbic Acid*—A possible alternative explanation for the above observations in the experiments with  $\text{H}_2\text{S}$  is that the equilibrium between dehydroascorbic acid and diketogulonic acid readjusts itself very slowly in the acid solutions in which the  $\text{H}_2\text{S}$  was used. Hence, although the equilibrium is disturbed by the conversion of the existing dehydroascorbic acid to ascorbic acid, very little more dehydroascorbic acid and thence ascorbic acid are formed from the diketogulonic acid.

This explanation is implicit in the description given by Herbert *et al.* (10) of the changes which they observed in neutral, alkaline, and dilute mineral acid solutions (anaerobic) of dehydroascorbic acid. They followed, among other changes, the mutarotation of these solutions, and found that nearly the same final rotatory power was attained in the alkaline and in the acid solutions. They designated the final mixture as an equilibrium mixture. They also referred to the slowness with which the lactone ring of dehydroascorbic acid is reconstituted in acid solution.

This alternative explanation was excluded by experiments with glutathione (these are described in more detail in a later section).

At pH 7 and more alkaline solutions, where the irreversible change in dehydroascorbic acid is very rapid, glutathione quickly reduces the latter substance to ascorbic acid, and has no such effect at this or at any other pH on the products of its irreversible change. Therefore, for all practical purposes, we may designate the change in dehydroascorbic acid as an irreversible one. The results of an experiment carried out at pH 7 are shown in Table III.

The technique used in this experiment was as follows: 3.5 cc. of a phosphate buffer at pH 7 were transferred to the lower part of

TABLE III

*Inability of Glutathione (800 Mg. Per Cent) to Regenerate Ascorbic Acid from Products of Irreversible Change in Dehydroascorbic Acid (10 Mg. Per Cent), at pH 7.0, and 37.5°, in Vacuo*

| Incubation of oxidised ascorbic acid |                               | Fraction of original oxidised ascorbic acid recovered in reduced state |
|--------------------------------------|-------------------------------|--|
| Before mixing with glutathione       | After mixing with glutathione |  |
| min.                                 | min.                          | per cent   |
| 0                                    | 30                            | 90   |
| 5                                    | 30                            | 70   |
| 15                                   | 30                            | 36   |
| 30                                   | 30                            | 14   |
| 60                                   | 30                            | 5  |
| 90                                   | 30                            | 2  |
| 120                                  | 30                            | <1   |
| 120                                  | 60                            | <1   |
| 120                                  | 120                           | <1   |
| 120                                  | 240                           | <1   |
| 120                                  | 1440                          | <1   |
| 120                                  | 2880                          | <1   |

a Thunberg tube. The solution was then frozen by immersing the tube in an alcohol-solid CO<sub>2</sub> bath. 1 cc. of a dehydroascorbic acid solution (formed by oxidation of ascorbic acid with I<sub>2</sub>) was next added above the frozen cake, and then frozen in the same way. The glutathione (dry) and 0.5 cc. of buffer solution were placed in the overhang. After the tube was thoroughly evacuated the ice cake was allowed to thaw, and pumping continued for 2 minutes after it had entirely melted. The tubes were then set away in a water bath at 37.5°. The contents of the upper and lower compartments were kept separate for different lengths of



time indicated in Table III. They were then mixed and the incubation continued as indicated in Table III. At the end of the incubation the vacuum was broken, the solution immediately acidified with metaphosphoric acid to a final concentration of 2 per cent, and the ascorbic acid present estimated by titration with 2,6-dichlorophenol indophenol. Controls with dehydroascorbic acid and glutathione alone were also carried through for the maximum incubation periods.

The figures in Table III show that as the period of incubation of the oxidized ascorbic acid solution at pH 7 was prolonged prior to mixing with the glutathione, the yield of ascorbic acid subsequently obtained diminished. This experiment provides further evidence that the irreversible change in dehydroascorbic acid is not an oxidation. The reconstitution of the lactone ring of dehydroascorbic acid, if the above formulæ are correct, evidently is as difficult in neutral as in acid solution.

Glutathione does disturb the course of the reactions, and possibly an equilibrium, among the irreversible products of dehydroascorbic acid. The control solution which contained dehydroascorbic acid initially and to which no glutathione was added invariably became brownish yellow after several hours. The solution to which glutathione was added after 2 hours incubation, *i.e.* after nearly all the dehydroascorbic acid had undergone its irreversible change, remained colorless even after 48 hours. Yet neither solution gave any appreciable titration with the dye on acidification.

*Ionization Constants of Dehydroascorbic Acid and of Diketogulonic Acid*—Another evidence of the irreversible change in dehydroascorbic acid is the resulting increase in strength of the acid group. This affords striking visual evidence of the transformation. It can be demonstrated by the following colorimetric procedure we have employed to measure the ionization constants of dehydroascorbic acid and of diketogulonic acid.

1 cc. of an aqueous 0.01 M solution of ascorbic acid oxidized with iodine was measured into the overhang of a Thunberg tube. The main (lower) compartment contained a suitable pH indicator, and a quantity of alkali equivalent to the HI formed in the oxidation of the ascorbic acid, plus an additional quantity which was varied from 0.1 to 0.8 mole equivalent of the ascorbic acid in the overhang.

Before the contents of the upper and lower compartments were mixed the Thunberg tubes were thoroughly evacuated. After being mixed, the initial pH of the solution was estimated by comparing the color immediately after mixing with the color of the same dye in standard buffer solutions. With thymol blue and phenolphthalein the color changes in a few seconds from the alkaline to the acid color following the formation of the more strongly acid irreversible product of dehydroascorbic acid. By successive trials the color formed immediately on mixing was bracketed more and more closely within those of the series of buffer standards.

The estimated initial pH values extended over a range from 8.05 to 9.2. These yielded the value of  $pK = 9.0 \pm 0.1$  for the ionizable hydrogen of dehydroascorbic acid. This hydrogen ion probably arises from an enolic group.

The first acid dissociation constant of diketogulonic acid was determined in the same manner except that the final, equilibrium colors were measured. About 24 hours were required at 25° for the attainment of this stage. The acidities of the different solutions fell within the pH ranges of brom-cresol green and brom-phenol blue, yielding a  $pK$  of approximately 3.3. The group involved here is probably a carboxyl group.

We obtained some indication here of a second ionizable hydrogen, with a  $pK$  between 7 and 8. We were unable to measure it more precisely with the above technique because all the dyes usable in the appropriate pH range—brom-thymol blue, chlorophenol red, phenol red, neutral red, and brom-cresol purple—were attacked by the oxidized ascorbic acid when more than 1 equivalent of additional base was added. As a result the color intensity or hue was changed. However, even in the changed state these dyes responded to changes in pH and so permitted a rough guess of the final pH. But it is possible that this ionizable hydrogen arises from some secondary oxidation product of diketogulonic acid (after reaction with the dye) rather than from this substance itself.

*Colorimetric Observations on Three Oxidation Stages of Ascorbic Acid*—When the reducing action of solutions of ascorbic acid and of dehydroascorbic acid are studied by colorimetric methods over a pH range from 2 to 9, the three oxidation stages of ascorbic acid

are clearly shown. The first, that of ascorbic acid to dehydroascorbic acid appears without the intervention of the second and third steps in the pH range from 2 to 4. The second, that of diketogulonic acid to an unstable intermediary, which in an alkaline reaction breaks down to *l*-threonic acid and oxalic acid, can be isolated in the pH range between 5.5 and 7.5 by beginning with dehydroascorbic acid. The actual reductant here is diketogulonic acid, arising in this pH range from the irreversible change in dehydroascorbic acid. The third oxidation stage occurs only in alkaline reactions. We can only guess at the substance which is oxidized. It seems that it is probably *l*-threonic acid.

The usual Thunberg vacuum technique was employed in these experiments with a series of reversible oxidation-reduction dyes ranging from *o*-cresol indophenol to methylene violet, and a series of buffers (McIlvaine's series) spaced at 0.5 pH intervals from pH 2 to 9. At each pH with each dye the reducing action of ascorbic acid and of dehydroascorbic acid was observed. The degree of reduction of the dye was estimated by comparison with known dilutions of oxidized dye. Where the reduction did not go to completion, it was taken as an equilibrium value when the degree of reduction observed remained unchanged for 24 to 48 hours, and not more than 2 to 3 days were required for the attainment of this value.

The details of the procedure were as follows: Aqueous stock solutions of the following dyes given in descending order of their oxidation-reduction potentials, *o*-cresol indophenol, thionine, methylene blue, indigotetra-, indigodi-, and indigomonosulfonate, brilliant alizarin blue, and methylene violet, were diluted with the buffer solution used to a final concentration of 0.00005 M. 2 cc. of this solution were pipetted into the lower compartment of a Thunberg vessel, 1 cc. of an aqueous solution of 0.005 M ascorbic or of dehydroascorbic acid into the overhang. Where ascorbic acid was used, HCl and KI equivalent to the HI present in the solution of dehydroascorbic acid were added. The vessels were evacuated as in the electrometric measurements, mixed, and then set away in water baths. At each pH two series of experiments were carried out, one at 37°, the other at 25°. The reduction rates were of course slower at the lower temperature, but otherwise the

results were essentially the same as those at 37°. We shall give the details only of the observations at the higher temperature.

**Colorimetric Measurement of Reducing Property of Ascorbic Acid. First Oxidation Stage**—Ascorbic acid rapidly completely reduced *o*-cresol indophenol and thionine over the whole pH range. With methylene blue equilibrium was attained at pH 4.5, and with indigotetrasulfonate at pH 2.5, 3.0, 3.5, and 4.0. The computed  $E'_0$  values are given in Table IV. At higher pH values reduction of these two dyes was eventually complete, or nearly so. This was the result of the intervention of the more powerful reducing

TABLE IV  
*Reversible Oxidation-Reduction Potentials of Ascorbic Acid. Relation between  $E'_0 \left( \frac{(\text{Reduced})}{(\text{Oxidized})} = 1 \right)$  and pH*

| pH  | First oxidation stage         |                |              | Second oxidation stage |                 |
|-----|-------------------------------|----------------|--------------|------------------------|-----------------|
|     | Electrometric                 |                | Colorimetric | 35°, authors           | 25°, Fruton (9) |
|     | 35°, Borsook and Keighley (7) | 30°, Ball (13) | 37°, authors |                        |                 |
| 2.5 | +0.252                        | +0.242         | +0.235       |                        |                 |
| 3.0 | +0.223                        | +0.212         | +0.206       |                        |                 |
| 3.5 | +0.193                        | +0.184         | +0.185       |                        |                 |
| 4.0 | +0.166                        | +0.158         | +0.155       |                        |                 |
| 4.5 | +0.145                        | +0.136         | +0.146       |                        |                 |
| 5.0 | +0.127                        | +0.118         |              |                        |                 |
| 5.5 | +0.112                        | +0.102         |              | +0.015                 | +0.019          |
| 6.0 |                               |                |              | -0.031                 | -0.021          |
| 6.5 |                               |                |              | -0.068                 | -0.051          |

action of the products of the irreversible change in the dehydroascorbic acid which arises from the oxidation of the ascorbic acid by the dyes. There was a negligible or no reduction of the dyes more negative than indigotetrasulfonate.

**Colorimetric Measurement of Reducing Property of Solutions of Dehydroascorbic Acid. Second Oxidation Stage**—The dehydroascorbic acid solutions did not reduce any of the dyes in 24 hours at hydrogen ion concentrations from pH 2.5 to 4.0 inclusive. At pH 4.5 between 5 and 10 per cent of *o*-cresol indophenol, thionine, methylene blue, and indigotetrasulfonate were reduced in 24 hours. Above this pH the reduction of these dyes was much more rapid.

*o*-Cresol indophenol was completely reduced at pH 5 in 4 hours, at pH 6.0 in 45 minutes, at pH 7 in 6 minutes, and at pH 8 in 3 minutes. The reduction of thionine and methylene blue was slower. At pH 5, 30 hours elapsed before the reduction of the thionine was complete, and 44 hours with methylene blue. At pH 6.0 the reduction times for these two dyes were 2.5 and 12 hours; at pH 7.0, 17 and 55 minutes; at pH 8.0, 4 and 14 minutes. With indigodisulfonate equilibrium degrees of reduction were attained at pH 5.5, 6.0, and 6.5. The computed  $E'_0$  values are given in Table IV. Indigomonosulfonate was only slightly reduced in 100 hours at pH 6.5, it was 75 per cent reduced in this time at pH 7.0, completely reduced in 3 hours at pH 8.0, and in 5 minutes at pH 9.0. Brilliant alizarin blue was not reduced below pH 7. At this pH the reduction was less than 10 per cent in 10 hours. It was 100 per cent in 2 hours at pH 8.0, and in 25 minutes at pH 9.0. There was no reduction of methylene violet even at pH 9.0.

The first point to be noted in these observations is the proof that dehydroascorbic acid can become a reducing agent. This property first appeared at pH 4, and then became progressively more conspicuous with increasing pH. With the most positive dye used, *o*-cresol indophenol, the reduction times with the solutions of dehydroascorbic acid were always greater than with those of ascorbic acid. Thus from pH 2.5 to 4, the two times were infinity and less than 1 minute respectively; at pH 5, 100 and < 1 minute; at pH 6, 45 and < 1 minute; at pH 7, 6 and < 1 minute; and pH 8, 3.5 and < 1 minute. These differences in reactivity of the reduced and oxidized forms make it possible to determine vitamin C by titrimetric methods with indophenols, and are some of the reasons for the empirically discovered superiority of the titration at pH 2.5 (15), to that at or near neutrality, as it was first done by Tillmans (16).

The failure of solutions of dehydroascorbic acid to reduce any of the dyes at acidities greater than at pH 4 was not solely because its conversion to diketogulonic acid is too slow here. Diketogulonic acid itself is inactive as a reducing agent below pH 4. We have carried out experiments in which the transformation of dehydroascorbic acid to diketogulonic acid was allowed to proceed to completion at pH 7, 8, and 9. The solutions were then acidified, and no reducing power was found.

The second point is that the reducing power of solutions of

dehydroascorbic acid coincides with respect to the pH at which it first appears and to its development with increasing pH, with the potential drift in the electrometric measurements, and with the loss in the property of reduction to ascorbic acid by  $\text{H}_2\text{S}$ . These three independent pieces of evidence attest in complete accord to the spontaneous change in dehydroascorbic acid. The fact that the latter substance, the oxidant of the first oxidation stage, becomes a more powerful reducing agent (by 0.1 volt at pH 5.5 for example, Table IV) than ascorbic acid, is further proof that the change is an irreversible one. This is the explanation for the inability of ascorbic acid to reduce dyes which are reduced under the same conditions by solutions which consisted initially solely of dehydroascorbic acid; *e.g.*, indigodisulfonate at pH 7. If the dye is too negative to be reduced by ascorbic acid, no dehydroascorbic acid appears, and hence no diketogulonic acid is formed, which is the actual reductant here.

We have pointed out above that there are two possible formulations for the second oxidation stage. One is diketogulonic acid to an intermediary which breaks down in alkaline reactions to *L*-threonic acid and oxalic acid; the other is diketogulonic acid directly to *L*-threonic acid and oxalic acid. Experiments, which space limitations do not permit us to discuss here, favor the first of these two alternatives.

*Colorimetric Observations on Third Oxidation Stage*—At pH 9.0 the dehydroascorbic acid solutions quickly completely reduced all the dyes used except methylene violet. The complete reduction of indigomonosulfonate and brilliant alizarin blue would not be expected from an extrapolation of the equilibrium values obtained with dehydroascorbic acid (transformed to diketogulonic acid) and indigodisulfonate at pH 5.5 to 6.5 inclusive.

Our interpretation of this second increase in reducing power is that it is a third oxidation stage, *i.e.* the oxidation of a product of the oxidation of diketogulonic acid, rather than the oxidation of a transformation product of diketogulonic acid, *i.e.* a different second oxidation stage.

This interpretation is supported by the following observations in the literature. Heard and Welch (17) found that at pH 7.36 and 38° 1 molecule of ascorbic acid took up 3 atoms of oxygen. Similarly Herbert *et al.* (10) observed that further oxidation of

ascorbic acid occurred after potassium permanganate equivalent to 2 atoms of oxygen had been used. Fruton (9), who studied the second oxidation stage, noted that at pH levels above 7.5 "the equilibria obtained were less reproducible and did not accord with the 0.06 slope fitting the readings in the more acid region."

In view of this uncertainty regarding the mechanism of the third oxidation stage, it is not worth while even to guess at its oxidation-reduction potential. It is sufficient to note that it is much more negative than the second oxidation stage.

In Table IV are collected the  $E'_0$  values we have obtained for the first and second oxidation stages. Our values for the first oxidation stage are in reasonably good agreement with those recently reported by Ball who employed a different electrometric method from ours.

We have assigned the oxidation-reduction potentials of ascorbic acid reported by Fruton to the second oxidation stage, although the implication in the communication in which they appeared is that they pertain to the first oxidation stage. The initial material in Fruton's experiments was ascorbic acid. It was oxidized *in vacuo* by dyes; and  $E'_0$  values for ascorbic acid were computed from the observed equilibrium degrees of reduction of these dyes whose oxidation-reduction potentials are known. The justification for our interpretation of his data is as follows: The pH range in which reproducible and concordant potentials were obtainable in these experiments was from 5.7 to 7.2. This is the range in which it is not possible by measuring the equilibrium state to obtain the reversible potentials of the first oxidation stage because of the transformation of dehydroascorbic acid to diketogulonic acid; and it is precisely the range of stability of the product of this second oxidation stage. The time required for the attainment of equilibrium in Fruton's experiments varied from 1.2 to 18 days (at 25°). The data in Tables II and III show that most of the dehydroascorbic acid formed in the first oxidation stage must have undergone its irreversible transformation in this time. The potential of the first oxidation stage is 100 millivolts more positive than that given by Fruton. On this point observers in three different laboratories using three different methods are now in accord. Further, both the theoretical and experimentally observed values for  $-dE'_0/dpH$  for the first reversible oxidation are 30 millivolts per

pH unit in the range from pH 5.5 to 7.0 instead of 60 millivolts as found by Fruton. Finally the  $E'_0$  values we obtained for the second oxidation stage, where the initial material was dehydroascorbic acid, are of the same order of magnitude as those given by Fruton (Table IV).

The dehydroascorbic acid used in all of the foregoing experiments was obtained by oxidation of ascorbic acid with iodine. Shwachman, Hellerman, and Cohen (18) have suggested that the oxidation of ascorbic acid by iodine may be atypical. This suspicion was aroused by their observation of greater reducing power in solutions containing ascorbic acid than our values for the potentials for the first reversible oxidation indicated. The explanation for their observations is that their experiments were carried out on the alkaline side of neutrality, where not only the first oxidation stage, but also the second and third more negative reductions occur. We have studied the properties of solutions of ascorbic acid oxidized by oxygen catalyzed by copper, charcoal, 2,6-dichlorophenol indophenol, bromine, and ferricyanide. The same reversible and irreversible changes were observed as when the oxidation was carried out with iodine.

### *Nutrition Experiments*

Dehydroascorbic acid possesses nearly the same antiscorbutic potency as ascorbic acid (19-21). Oxidized solutions of the vitamin, particularly if these are the natural juice, lose their potency on standing. It has been assumed that this loss is the result of "irreversible oxidation." The experiments described in the foregoing section led us to question this interpretation.

Nutrition experiments were undertaken to examine this and two other questions: whether the reconversion of diketogulonic acid to dehydroascorbic, which cannot be done *in vitro* by means of  $H_2S$  in acid nor by glutathione in neutral or alkaline solution, can occur *in vivo*; and whether some mechanism exists *in vivo* which prevents or retards the transformation of dehydroascorbic acid to diketogulonic acid.

The general method and rationale of these experiments were as follows: Solutions containing initially the same amount of dehydroascorbic acid were incubated at different hydrogen ion concentrations. As a result of these differences in pH different amounts



of dehydroascorbic acid were transformed to diketogulonic acid. The same volumes of these solutions were then administered to guinea pigs whose only source of vitamin C was these solutions. The amount of dehydroascorbic acid remaining in each solution at the end of the incubation was determined by treatment with  $H_2S$  after acidification and estimation of the regenerated ascorbic acid by titration. The antiscorbutic potency of these solutions was tested by comparison with graded protective and subprotective doses of orange juice. If the antiscorbutic potency of the incubated solutions turned out to be greater than that of (the ascorbic acid equivalent of) the dehydroascorbic acid remaining at the time of administration to the animal, it would be evidence of the reversion of diketogulonic acid to dehydroascorbic acid; if it was much less, then there is no mechanism *in vivo* preventing or retarding the transformation of dehydroascorbic acid to diketogulonic acid; if it was nearly the same, then a mechanism does exist *in vivo* which protects dehydroascorbic acid from its irreversible transformation.

The last result was the one obtained.

The standard technique described by Sherman and Smith (22) was used in these experiments. Two sets were carried out: one in which the incubation of the dehydroascorbic acid solutions until the moment of administration to the animal took place *in vacuo*, the other in air. Dehydroascorbic acid was prepared by oxidizing solutions of ascorbic acid with iodine. Aliquots were then brought to pH 4.0, 6.0, 7.0, and 9.0 by means of buffers. These were then incubated for an hour either in air or *in vacuo*. The following amounts of dehydroascorbic acid (expressed in terms of ascorbic acid) remained at the end of this time, in the solutions at pH 4.0, 6.0, 7.0, and 9.0 respectively, 1.4, 1.2, 0.75, and 0.2 mg. per cc. In the series where the incubation took place *in vacuo* the mixing with the buffer occurred after the tubes were evacuated. All these solutions were prepared freshly each day. A volume of one of these containing originally 1.5 mg. of ascorbic acid was administered daily to each animal either subcutaneously or by mouth.

The controls were the basal ration alone, and supplemented daily with one of the following: 3 cc. of orange juice, 1.5 mg. of reduced ascorbic acid, the buffer solutions, an amount of KI equal

to that given in the solutions of oxidized ascorbic acid, these with and without buffer, and with and without 3 cc. of orange juice. Where it was possible two sets of similar controls were carried, one in which the control solution was given subcutaneously, in the other by mouth.

Each experimental and control group consisted of five animals.

The method of assay was based on the scurvy score of each animal determined post mortem either after the animal had died or was killed. The following stigmata of the disease were appraised: the extent and number of hemorrhages, the beading and enlargement of the costochondral junctions, the softness of the mandibles, scapulæ, and ribs, and the looseness of the incisors.

These experiments showed that the antiscorbutic potency of the oxidized and incubated solutions of ascorbic acid was quantitatively very nearly equal to the amount of the reduced form which could be recovered from them by treatment with  $H_2S$ . The same results were obtained whether the solutions were incubated *in vacuo* or in air, and whether these solutions were administered subcutaneously or by mouth.

The fact that these solutions were slightly less antiscorbutic than the ascorbic acid equivalent of the dehydroascorbic acid which they contained was probably the result of transformation of the latter substance to diketogulonic acid and other products before it reached its site of action in the tissues.

We may conclude from these experiments that diketogulonic acid and its transformation and oxidation products possess no antiscorbutic potency. The tissues resemble  $H_2S$  and glutathione in that they are unable to convert these substances to dehydroascorbic or ascorbic acid. This point is interesting because it is possible to recover 75 per cent or more of ascorbic acid from the products of the irreversible transformation of dehydroascorbic acid by concentrating their acid solution to a syrup in the presence of  $H_2I$ . This procedure therefore has no physiological analogue.

In a recent communication Roe and Barnum (23) reported that dehydroascorbic acid possesses only one-quarter the antiscorbutic potency of ascorbic acid. This observation is in disagreement with those of all other workers who have investigated this point (19-21) and with our own observations. For example we found no sign of scurvy in guinea pigs receiving for 60 days 1.2 mg. of

dehydroascorbic acid as their only source of vitamin C, and only moderate scurvy after the same length of time in animals receiving 0.75 mg. of dehydroascorbic acid. 1 mg. of ascorbic acid daily is the minimum protective dose for the standard animals used in these experiments.

The explanation for this discrepancy is that Roe and Barnum neutralized their solutions of oxidized ascorbic acid before administering them. They were misled by their method of analysis, boiling with HCl containing  $\text{SnCl}_2$ , and determination of the resulting furfural, which indicated erroneously that dehydroascorbic acid remains unchanged in neutralized solutions for as long as 3 hours after its preparation. (See Table II.)

We attempted to analyze the blood, liver, and adrenals of the experimental animals for ascorbic and dehydroascorbic acids in order to observe whether the tissues reduce the latter to the former substance. The ascorbic acid was titrated in salicylic or metaphosphoric acid filtrates with 2,6-dichlorophenol indophenol. To our surprise we were unable to attain an end-point at all comparable to that apparently obtained by all other workers with guinea pig tissues, or by ourselves with the plasma of seven other animal species, human urine, rat liver, intestine, kidney, and adrenal, and plant tissues. There appeared to be a reducing substance in the blood, liver, and adrenals of all the guinea pigs in our experiments which did not reduce the dye as quickly as ascorbic acid, but did so quickly enough to prevent us from obtaining an end-point in which we had any confidence. The value obtained with the dye titration depended too much on the speed of titration. This interfering substance reduced iodine, and was not removed by the mercury precipitation of Emmerie and van Eekelen (24). A relatively enormous quantity was present. In the following section conclusive evidence is presented that human and rat tissues convert dehydroascorbic acid to the reduced form.

Fox and Levy (21) administered reversibly oxidized ascorbic acid to guinea pigs and found large enough amounts of the reduced form in the tissues after 60 days to make it seem certain that dehydroascorbic acid is reduced in guinea pig tissues. Apparently they did not encounter, or were not hindered by, the reducing substance which marred our titrations.

*Physiological Experiments*

The nutrition experiments showed that dehydroascorbic acid is protected *in vivo* from rapid transformation to the antiscorbutically impotent diketogulonic acid. We sought by some physiological experiments to locate the site of this protection of dehydroascorbic acid, and to obtain some information regarding its mechanism. The experiments consisted in following the fate of dehydroascorbic acid and of ascorbic acid when these were added to blood, plasma, minced and intact isolated tissues, and also after their ingestion.

*Analytical Methods*—The following analytical methods were used. Ascorbic acid in blood, tissues, and urine, was titrated with 2,6-dichlorophenol indophenol after acidification with metaphosphoric acid. According to the volume of solution to be titrated the titration was carried out with a capillary burette (25), or a semimicroburette containing 1 cc. in a length of 45 cm., or in a sugar burette. Ascorbic acid cannot be recovered quantitatively from plasma or tissue filtrates after precipitation with trichloroacetic acid, sulfosalicylic acid, or sodium tungstate and sulfuric acid. It can be recovered quantitatively from metaphosphoric acid blood and tissue filtrates (26). Accordingly metaphosphoric acid was used (in a final concentration of 2 to 4 per cent) both for purposes of acidification only—with urine or solutions of ascorbic acid and glutathione—and for protein precipitation and acidification with blood and tissues.

In urine only a fraction of the reducing material titratable with 2,6-dichlorophenol indophenol is ascorbic acid. Among the other reducing substances are polyphenols such as homogentisic acid, pigments, and chromogens (27). The pigments, and probably other reducing substances, can be removed from urine acidified with sulfosalicylic or metaphosphoric acid by shaking for a few minutes with Lloyd's reagent—100 mg. of Lloyd's reagent for 10 cc. of solution. At the same time about 10 per cent of the ascorbic acid present is removed. The total amount of dye-reducing material removed in this way varied, expressed in terms of ascorbic acid, from 0.6 to 6 mg. per 100 cc. of urine. These quantities were negligible for our comparative purposes, especially as Barrenscheen and his collaborators had shown that the excretion of this fraction was fairly constant in any one individual. The figures for urine

given below are those for urine not treated with Lloyd's reagent. The conclusions were identical with the values obtained after treatment with Lloyd's reagent.

Dehydroascorbic acid was determined by treating the metaphosphoric acid filtrates with  $H_2S$  in the usual manner: a stream of wet  $H_2S$  was passed through the solution for 3 hours, the material was left standing overnight in a stoppered flask filled with  $H_2S$ , and this treatment was followed by removal of the  $H_2S$  by a vigorous stream of wet nitrogen for 3 to 5 hours.

When whole blood (beef, cat, dog, human, pig, rat, and sheep) is precipitated with either trichloroacetic acid, sulfosalicylic acid, sodium tungstate plus sulfuric acid, or metaphosphoric acid, nearly all the ascorbic acid whether initially present, or added, is oxidized to dehydroascorbic acid. Similar precipitation of the plasma or serum leaves practically all the ascorbic acid in its reduced state. Our experience here differs from that of Kellie and Zilva (28) who reported complete recovery of added ascorbic acid in this form after precipitating whole blood or a buffer solution containing red blood corpuscles with trichloroacetic acid. Their experience is the more surprising as they used trichloroacetic acid as precipitant. Confirming Fujita and Iwatake (26) we have been unable to recover added ascorbic acid quantitatively as such even when added to serum, when trichloroacetic acid was used as precipitant. Van Eekelen and his collaborators (29) have reported findings similar to ours—when whole blood is treated with an acid protein precipitant, nearly all of the ascorbic acid present is oxidized to dehydroascorbic acid. These authors accordingly revised their earlier opinion that a considerable fraction (if not all) of the ascorbic acid in human blood is in the reversibly oxidized form. Their present conclusion, that of Kellie and Zilva (30), of Farmer and Abt (31), and ours, is that practically all (if not all) of the ascorbic acid in plasma and serum is in the reduced state. This conclusion differs, with respect to human blood, from that of Plaut and Bülow (32) who found most of the ascorbic acid in serum in the form of dehydroascorbic acid.

*Stability of Ascorbic Acid and of Dehydroascorbic Acid in Blood and in Plasma*—Table V is a summary of a typical group of experiments in which the stability of ascorbic acid and of dehydroascorbic acid in Ringer's solution, plasma, and whole blood was studied.

The data show the gradual disappearance of added ascorbic acid from all three solutions. The rate of this disappearance was nearly the same in Ringer's solution and in plasma, approximately half remaining after 4 hours. In whole blood, *i.e.* in plasma in contact with its corpuscles, the vitamin was much more stable.

TABLE V

*Stability of Ascorbic Acid and Dehydroascorbic Acid in Ringer's Solution, Plasma (Human), and Whole Blood (Human), under 95 Per Cent Oxygen and 5 Per Cent CO<sub>2</sub> at 37.5°*

| Solution  | Concentration and form of added ascorbic acid |                       | Ascorbic acid, mg. per cent, found after |          |       |          |          |        |
|---|---|-----------------------|--|----------|-------|----------|----------|--------|
|   |   |                       | Form                                     | 0.75 hr. | 1 hr. | 1.5 hrs. | 2.5 hrs. | 4 hrs. |
| Ringer's,<br>pH 7.4<br>Plasma                   | <i>mg. per cent</i>                           |                       |  |          |       |          |          |        |
|   | 12  | Ascorbic acid         | Reduced                                  | 9.0      |       | 7.6      | 6.9      | 5.9    |
|   |   |                       | After H <sub>2</sub> S                   | 9.9      |       | 8.4      | 7.7      | 6.5    |
|   | 0   |                       | Reduced                                  | 1.0      |       | 0.6      | 0.3      | 0.2    |
|   |   |                       | After H <sub>2</sub> S                   | 1.7      |       | 0.8      | 0.6      | 0.5    |
|   | 40  | Dehydro-ascorbic acid | Reduced                                  | 1.5      |       | 2.0      | 2.4      | 2.5    |
|   |   |                       | After H <sub>2</sub> S                   | 3.7*     |       | 3.8*     | 4.3*     | 4.8*   |
|   | 19.4  | Ascorbic acid         | Reduced                                  | 16.3     |       | 14.1     | 11.9     | 8.8    |
| Whole<br>blood,<br>plasma<br>only ana-<br>lyzed |   |                       | After H <sub>2</sub> S                   | 18.6     |       | 14.7     | 12.9     | 10.3   |
|   | 0   |                       | Reduced                                  | 1.2      |       | 1.2      | 0.8      | 0.8    |
|   |   |                       | After H <sub>2</sub> S                   | 1.5      | 1.9†  | 1.3      | 1.3      | 1.3    |
|   | 20  | Dehydro-ascorbic acid | Reduced                                  | 1.5      |       | 2.7      | 3.6      | 4.9    |
|   |   |                       | After H <sub>2</sub> S                   | 3.0*     | 9.7†  | 4.5*     | 4.3*     | 5.9*   |
|   | 12  | Ascorbic acid         | Reduced                                  | 21.0     |       | 19.3     | 18.2     | 17.1   |
|   |   |                       | After H <sub>2</sub> S                   | 20.1     | 9.4†  | 18.9     | 19.6     | 19.0   |
|   |   |                       |  |          |       |          |          | 14.6   |

\* In these solutions the rate of reduction of the dye was definitely slower than in the others, or in plain aqueous acid solutions of ascorbic acid. The end-points, once attained, were quite stable. It is questionable whether these figures may be taken as ascorbic acid.

† Figures for whole blood precipitated as such. Filtrate treated with H<sub>2</sub>S.

Nearly three-quarters was recovered in the reduced form after 4 hours. (Nearly all of the ascorbic acid added to blood remains in the plasma; hence the figure of 20.1 mg. per cent when the amount added gave a concentration in whole blood of only 12 mg. per cent.) We have frequently found greater stability in whole blood over a period of 2 hours than is recorded here. In both

human and rat blood we have recovered 95 to 97 per cent of the ascorbic acid added in the reduced form.

The preexisting ascorbic acid in plasma and in whole blood exhibited the same difference in stability as the added ascorbic acid. The figures show also that only a small fraction of the ascorbic acid (whether preexisting or added) which had disappeared was found in the reversible oxidized form. There is, therefore, no effective mechanism in the blood for protecting dehydroascorbic acid.

These observations on the greater stability of ascorbic acid in whole blood than in plasma are in accord with those of Kellie and Zilva (28) on the protective influence of red blood corpuscles added to buffer solutions. De Caro and Giani (33) record a lower stability of ascorbic acid in defibrinated guinea pig blood than in serum. Their result is probably an artifact of their analytical procedure—precipitation of the whole blood and serum with trichloroacetic acid.

When dehydroascorbic acid was added to the blood, Table V shows that after  $\frac{3}{4}$  hour only 4 per cent was recovered as ascorbic acid by subsequent treatment with  $\text{H}_2\text{S}$ . This finding and those after longer incubation periods show that dehydroascorbic acid is not reduced at any significant rate in blood or plasma; and also, in accord with the conclusion drawn from the fate of preexisting or added ascorbic acid, that there is no effective mechanism in blood for the protection of dehydroascorbic acid. This substance is transformed to diketogulonic acid as rapidly in blood as in a buffer solution at this pH (Table III).

We have drawn these conclusions although, as Table V shows, we observed a slow increase in reducing material after the addition of dehydroascorbic acid to plasma and to whole blood. We doubt whether this material is ascorbic acid. The filtrates in which it was contained reduced the dye more slowly than solutions of ascorbic acid. We found no evidence of a mechanism in blood capable of reducing either preexisting or added dehydroascorbic acid. The low recovery of ascorbic acid after treatment with  $\text{H}_2\text{S}$  from plasma and blood to which dehydroascorbic acid had been added also argues against this reducing material being ascorbic acid.

Plaut and Bülow (32) came to the conclusion from *in vitro* experi-

ment with serum, and *in vitro* and injection experiments with cerebrospinal fluid that dehydroascorbic acid is not reduced in either of these fluids *in vitro* or *in vivo*.

Roe and Barnum (23) deduced from evidence similar to that in Table V the existence of an enzyme in the blood, which is capable of reducing dehydroascorbic acid. The interpretation of their data is uncertain because the results are expressed as titration figures. They added 100 mg. per cent of dehydroascorbic acid and incubated the blood or plasma for 3 hours at 38°. When their titration figures are converted to mg. per cent on the assumption that their figures for plasma represent the normal ascorbic acid content, the fraction of the added dehydroascorbic acid which they recovered as ascorbic acid (without H<sub>2</sub>S treatment) was less than in our experiments. Their data and those in Table V are therefore in accord in that the hypothetical mechanism in the blood for the reduction of dehydroascorbic acid, if it exists at all, reacts too slowly to save most of the dehydroascorbic acid from irreversible transformation.

The question whether or not the reducing material which slowly appears after the addition of dehydroascorbic acid to blood is ascorbic acid must be left until some such method as ultraviolet spectroscopy is applied to the problem.

*Distribution of Ascorbic Acid between Blood and Corpuscles*—Table VI shows that in seven animal species the bulk of added ascorbic acid does not enter the corpuscles. No allowance was made in the figures given for oxidation of some of the added ascorbic acid, which certainly occurred. Only in the cases of the pig and sheep were there any large differences between the concentrations found in the plasma and those calculated on the basis of complete impermeability of the corpuscle, and no oxidation. But even in these two specimens the amounts found in the plasma were much larger than if there had been equipartition between plasma and corpuscles. This impermeability of the red blood corpuscle to ascorbic acid recalls the observation of Olmsted (34) that red blood corpuscles, particularly when the blood is not oxalated, are impermeable to glucose.

*Reduction of Dehydroascorbic Acid in Tissues*—Although dehydroascorbic acid is neither reduced to ascorbic acid, nor protected from its irreversible transformation to diketogulonic acid



in the blood, it is rapidly reduced in the tissues. The evidence supporting this conclusion is 3-fold. First, when dehydroascorbic acid is ingested, there are an increase in the ascorbic acid content of the plasma and a very large increase in the ascorbic acid excreted in the urine. On the other hand there are only negligible increases in the dehydroascorbic acid content of the plasma and

TABLE VI

*Concentration of Ascorbic Acid in Plasma after Its Addition to Whole Blood and Incubation for 1 Hour at 37.5°*

| Species | Anesthetic used | Preliminary treatment | Concentration in whole blood of added ascorbic acid | Ratio of serum volume to total blood volume X 100 | Ascorbic acid concentration in plasma                                |              | Degree of hemolysis |
|---------|-----------------|-----------------------|---|---|--|--------------|---------------------|
|         |                 |                       |   |   | Calculated on complete impermeability of corpuscles and no oxidation | Observed     |                     |
|         |                 |                       | mg. per cent  |   | mg. per cent   | mg. per cent |                     |
| Beef    | None            | Defibrinated          | 0   |   |  | 0.4          | 0                   |
| "       | "               | "                     | 15  | 65  | 23.5   | 20.6         | 0                   |
| Cat     | Ether           | "                     | 0   |   |  | 0.3          | 0                   |
| "       | "               | "                     | 10  | 69  | 14.7   | 13.3         | 0                   |
| "       | "               | Oxalated              | 0   |   |  | 0.2          | 0                   |
| "       | "               | "                     | 10  | 69  | 14.6   | 12.9         | 0                   |
| Dog     | None            | Defibrinated          | 0   |   |  | 0.5          | ++                  |
| "       | "               | "                     | 15  | 58  | 26.0   | 22.8         | ++                  |
| Human   | "               | Oxalated              | 0   |   |  | 1.2          | 0                   |
| "       | "               | "                     | 12  | 55  | 23.1   | 21.0         | 0                   |
| Pig     | "               | Defibrinated          | 0   |   |  | 0.6          | +                   |
| "       | "               | "                     | 15  | 50  | 30.6   | 24.8         | +                   |
| Rat     | Chloroform      | Oxalated              | 0   |   |  | 0.2          | +++                 |
| "       | "               | "                     | 10  | 63  | 15.6   | 12.8         | +++                 |
| Sheep   | None            | Defibrinated          | 0   |   |  | 0.9          | 0                   |
| "       | "               | "                     | 15  | 49  | 31.5   | 23.6         | 0                   |

urine. Second, dehydroascorbic acid added to minced or intact isolated tissues is rapidly reduced. This finding stands in marked contrast to the findings in blood. Third, the reducing mechanism in the tissues capable of effecting a rapid reduction of dehydroascorbic acid under physiological conditions was demonstrated to be probably glutathione.

*Changes in Ascorbic Acid and Dehydroascorbic Acid Content of Blood and Urine after Ingestion of Large Quantities of These Substances*—Typical results of one of a number of such experiments are given in Table VII.

Both the ascorbic acid and the dehydroascorbic acid were taken in the form of orange juice. The oxidized form was prepared by oxidizing the ascorbic acid in orange juice with charcoal (35). The amounts of ascorbic acid in the ordinary daily diets of the two subjects in this experiment were different. The only citrus fruits or juices in the diet of the subject who drank the untreated orange juice was one grapefruit every other day. The other subject took 300 to 400 cc. of orange juice daily. This dietary difference is reflected in the difference in the urinary excretion of ascorbic acid of the two subjects both before and after ingestion of the orange juice.

The figures in Table VII show that in both cases, *i.e.* after the ingestion of the reversibly oxidized and of the reduced forms of the vitamin, there were definite increases in the ascorbic acid in the plasma and urine, and only insignificant changes in the concentrations of oxidized ascorbic acid. Johnson and Zilva (36) had previously found increased amounts of reduced and no dehydroascorbic acid in the urine of human subjects after the ingestion of a large quantity of dehydroascorbic acid. The group of experiments of Table VII were carried out prior to our having seen the paper by these authors.

These experiments demonstrate the rapid reduction of dehydroascorbic acid in the animal body; and that only a very small fraction, if any, of the total ascorbic acid is transported as dehydroascorbic acid. Taken in conjunction with those in blood described above these findings indicate that the reduction of dehydroascorbic acid occurs in the tissues.

*Reduction of Dehydroascorbic Acid by Minced and Intact Isolated Tissues*—Table VIII shows that when dehydroascorbic acid is added to minced tissues it is rapidly reduced.

The procedure in this experiment was as follows: Rat kidney and liver were frozen with solid CO<sub>2</sub>, pulverized in the frozen state, triturated with an equal volume of phosphate buffer at pH 7.0, and then squeezed through cheese-cloth. To each 5 cc. of the juice 0.5 mg. of dehydroascorbic acid (in solution) was added. The

TABLE VII

*Concentrations of Reduced and Reversibly Oxidized Ascorbic Acid in Human Plasma and Total Urinary Excretion after Ingestion of 450 Cc. of Plain Orange Juice, and of Orange Juice in Which the Ascorbic Acid Was Oxidized with Charcoal*

|  |                      | Time after ingestion  |                    |                     |                    |                    |
|--|----------------------|---|--------------------|---------------------|--------------------|--------------------|
|  |                      | 0 hr.   | 1 hr.              | 2 hrs.              | 3 hrs.             |                    |
| Plain orange juice                               |                      |   |                    |                     |                    |                    |
| Plasma   | Ascorbic acid        | mg. per cent  | mg. per cent       | mg. per cent        | mg. per cent       |                    |
|  | Dehydroascorbic acid | 1.5   | 2.1                | 1.9                 | 2.0                |                    |
|  |                      | 0.2   | 0.2                | 0.15                | 0.1                |                    |
|  | Total                | 1.7   | 2.3                | 2.0                 | 2.1                |                    |
|  |                      | Urine excretion in 1 hr. intervals ending at following hrs. after ingestion |                    |                     |                    |                    |
| Urine  | Urine volume .....   | 0.5 hr. before<br>141 cc.   | 0.5 hr.<br>125 cc. | 1.5 hrs.<br>270 cc. | 2.5 hrs.<br>68 cc. | 3.5 hrs.<br>58 cc. |
|  |                      | mg.   | mg.                | mg.                 | mg.                | mg.                |
|  | Ascorbic acid        | 2.3   | 2.4                | 25.3                | 61.9               | 21.6               |
|  | Dehydroascorbic acid | 0.2   | 0.4                | 0.8                 | 0.4                | 0.8                |
|  | Total                | 2.5   | 2.8                | 26.1                | 62.3               | 22.4               |
| Orange juice in which ascorbic acid was oxidized |                      |   |                    |                     |                    |                    |
| Plasma   |                      | Time after ingestion  |                    |                     |                    |                    |
|  |                      | 0 hr.   | 1 hr.              | 2 hrs.              | 3 hrs.             |                    |
|  |                      | mg. per cent  | mg. per cent       | mg. per cent        | mg. per cent       |                    |
|  | Ascorbic acid        | 1.6   | 2.2                | 2.35                | 2.3                |                    |
|  | Dehydroascorbic acid | 0.3   | 0.3                | 0.4                 | 0.3                |                    |
|  | Total                | 1.9   | 2.5                | 2.75                | 2.6                |                    |
|  |                      | Urine excretion in 1 hr. intervals ending at following hrs. after ingestion |                    |                     |                    |                    |
| Urine  | Urine volume .....   | 0 hr.<br>250 cc.  | 1 hr.<br>340 cc.   | 2 hrs.<br>118 cc.   | 3 hrs.<br>75 cc.   |                    |
|  |                      | mg.   | mg.                | mg.                 | mg.                |                    |
|  | Ascorbic acid        | 26  | 76                 | 112                 | 139                |                    |
|  | Dehydroascorbic acid | 0   | 0                  | 3                   | 4                  |                    |
|  | Total                | 26  | 76                 | 115                 | 143                |                    |

juice was then incubated at 37.5° in air or *in vacuo* for 15 minutes. At the end of this time 2 cc. of 10 per cent metaphosphoric acid were added for each 5 cc. of juice. The ascorbic acid and glutathione content of the filtrate were then measured—the glutathione by the method of Quensel and Wacholder (37). The glutathione values obtained, referred to the original weight of tissue, are of the

TABLE VIII

*Reduction of Dehydroascorbic Acid by Minced Rat Tissues in Air and in Vacuo, at pH 7.0, and 37.5°, in 15 Minutes*

| Tissue                                       | Experiment carried out in air or in vacuo | Ascorbic acid in 5 cc. solution |                        | Reduced glutathione found in solution | Added dehydro-ascorbic acid recovered as ascorbic acid |                                   |
|--|---|---------------------------------|------------------------|---------------------------------------|--|-----------------------------------|
|  |   | Added as dehydro-ascorbic acid  | Found as ascorbic acid |                                       | Observed   | Calculated (see Tables X and XII) |
|  |   | mg.                             | mg.                    | mg. per cent                          | per cent   | per cent                          |
| Kidney                                       | Air                                       | 0                               | 0.27                   | 59                                    |  |                                   |
|  | "   | 0.5                             | 0.53                   | 60                                    | 52   | 35                                |
|  | Vacuum                                    | 0                               | 0.22                   | 76                                    |  |                                   |
|  | "   | 0.5                             | 0.51                   | 59                                    | 59   | 40                                |
| Liver  | Air                                       | 0                               | 0.35                   | 74                                    |  |                                   |
|  | "   | 0.5                             | 0.63                   | 70                                    | 60   | 50                                |
|  | Vacuum                                    | 0                               | 0.32                   | 80                                    |  |                                   |
|  | "   | 0.5                             | 0.58                   | 70                                    | 52   | 50                                |
| Intestinal mucosa thoroughly macerated       | Air                                       | 0                               | 0.33                   | 66                                    |  |                                   |
|  | "   | 0.5                             | 0.58                   | 44                                    | 50   | 30                                |
|  | Vacuum                                    | 0                               | 0.38                   | 58                                    |  |                                   |
|  | "   | 0.5                             | 0.64                   | 44                                    | 52   | 30                                |
| Intestinal mucosa not ground up, not diluted | Air                                       | 0                               | 0.43                   | 105                                   |  |                                   |
|  | "   | 0.5                             | 0.75                   | 105                                   | 64   | 55                                |

same order of magnitude as those determined by Benet and Weller (38) with their cadmium-iodate method. With intestinal mucosa one experiment was carried out with the tissue macerated as with liver and kidney; and a second in which the mucosa after being scraped off the muscular wall was left intact.

Table VIII shows that the reduction of dehydroascorbic acid by minced tissues is as rapid in air as *in vacuo*. This finding and

the rates of reduction observed indicate (anticipating here the results in Tables X and XII) that the reducing agent is glutathione. The figures for the calculated amounts given in Table VIII were obtained from rate studies with glutathione (see below). The reduction obtained in excess of these calculated amounts may be ascribed to the fixed —SH groups. A slow reduction of oxidized ascorbic acid by washed, minced muscle was observed by Szent-Györgyi (2). He ascribed this reducing action to the fixed —SH groups. The smallness of the excess of the observed over the calculated rate of reduction argues against the existence of an enzyme for this reaction. If there is an enzyme its potency or concentration is low.

Table IX shows the reduction of dehydroascorbic acid by slices or sections of surviving scorbutic guinea pig tissues in an atmosphere of 95 per cent oxygen and 5 per cent CO<sub>2</sub>.

The technique of preparing and handling the tissue slices has been described in a previous communication (39). The figures in Table IX are those obtained after subtracting the quantities found in the parallel controls, where the tissues were incubated without added dehydroascorbic acid. The small amounts of dehydroascorbic acid found may have been formed during the extraction of the tissues with acid water.

The reducing capacity of the tissues for dehydroascorbic acid is greater than the bare figures in Table IX indicate. In interpretation of these figures it must be borne in mind that the irreversible change in dehydroascorbic acid in Ringer's solution at the pH and temperature of these experiments is very rapid, and the volume of solution (5 cc.) in which the tissue slices were suspended was quite large. Unlike a minced tissue in which the reducing mechanism is uniformly distributed throughout the solution, in these experiments with tissue slices the reducing mechanism was concentrated in not more than 20 per cent of the volume, and was effective against only that small fraction of the dehydroascorbic acid which had succeeded in penetrating into the interior of the cell before undergoing irreversible change in the external solution.

*Protection of Ascorbic Acid from Oxidation by Minced and Intact Tissues*—De Caro (33), Mawson (40), and Kellie and Zilva (28) have shown that minced tissues and tissue extracts protect ascorbic acid from oxidation. Quastel and Wheatley (41) have observed a

similar protective action exerted by rat liver slices, and we have observed it with sections or slices of scorbutic guinea pig tissues—intestine, liver, and kidney.

The explanation given has been that the tissues combine with the heavy metals, iron and copper, which catalyze the oxidation of ascorbic acid. The experiments in Tables VIII and IX indicate that an additional protective mechanism, and possibly a more important one, is reduction of the dehydroascorbic acid formed by any oxidation of the ascorbic acid which may have occurred.

TABLE IX

*Reduction of Dehydroascorbic Acid (10 Mg. Per Cent) by Scorbutic Guinea Pig Tissue Sections in Ringer's Solution under 95 Per Cent Oxygen and 5 Per Cent CO<sub>2</sub> in 3 Hours, at pH 7.4, and 37.5°*

| Tissue                      | Dry weight | Amount recovered |                          | Recovered, reduced + oxidized |
|-----------------------------|------------|------------------|--------------------------|-------------------------------|
|                             |            | As ascorbic acid | As dehydro-ascorbic acid |                               |
|                             | mg.        | mg. per cent     | mg. per cent             | per cent                      |
| Ringer's solution . . . . . |            | 0.038            | 0.30                     | 3.5                           |
| Liver . . . . .             | 112        | 1.24             | 0.38                     | 16                            |
| Kidney . . . . .            | 122        | 1.36             | 0.06                     | 14                            |
| Intestine . . . . .         | 323        | 2.09             | 0.16                     | 23                            |

#### *Reduction of Dehydroascorbic Acid by Glutathione*

In his first full account of hexuronic acid Szent-Györgyi (2) reported that when the reversibly oxidized substance was added to a suspension of minced kidney, after 15 minutes a qualitative test for the reduced form was obtained, and at the same time the nitroprusside test had become weaker. Also when oxidized hexuronic acid was incubated *in vacuo* in neutral solution with glutathione, the latter substance was oxidized. The method used for reduced ascorbic acid, when applied to a minced tissue, is questionable. No mention was made of the ascorbic acid in the minced kidney preparation alone. It was possible that the oxidation of glutathione observed was the result of interaction with one of the products of the irreversible change in dehydroascorbic acid which is very rapid at pH 7.0. Later Pfankuch *et al.* (42) and Bersin and his collaborators (43) stated that neither glutathione alone nor

cysteine alone is able to reduce oxidized ascorbic acid. Pfankuch adduced some evidence for the existence of an enzyme in the potato which makes this reaction possible; and by implication Bersin and his collaborators postulated the existence of such an enzyme in animal tissues—at least in the guinea pig adrenal.

Preliminary to an investigation of this postulated enzyme we reexamined the reaction between glutathione and dehydroascorbic acid. We found that if a sufficiently high concentration of glutathione is used, the reduction of dehydroascorbic acid is very rapid,

TABLE X

*Reduction of Dehydroascorbic Acid, Initial Concentration 4 Mg. Per Cent, by Different Concentrations of Glutathione, in Vacuo, at pH 7.0, and 37.5°, in 4 Hours*

| Glutathione         | Oxidizing agent for ascorbic acid          | Reduction of oxidized ascorbic acid added |
|---------------------|--|---|
| <i>mg. per cent</i> |  | <i>per cent</i>                           |
| 4                   | I <sub>2</sub>                             | 3   |
| 20                  | "  | 13  |
| 60                  | "  | 40  |
| 100                 | "  | 57  |
| 150                 | "  | 72  |
| 200                 | "  | 88  |
| 200                 | Br <sub>2</sub>                            | 96  |
| 200                 | Air + CuCl <sub>2</sub> (36 mg. per liter) | 90  |
| 250                 | I <sub>2</sub>                             | 96  |
| 300                 | "  | 95  |
| 350                 | "  | 100                                       |
| 400                 | "  | 97  |

even in air. The reduction of this substance by minced tissues can be nearly quantitatively accounted for by the acid-soluble —SH groups (*i.e.*, mainly glutathione) on the assumption that no enzyme intervenes.

*Rate of Reduction of Dehydroascorbic Acid by Different Concentrations of Glutathione*—Table X shows the effect of different glutathione concentrations on the degree of reduction of the dehydroascorbic acid. The figures in Tables X to XIV require little additional comment.

The experimental and analytical technique used was as follows:

10 mg. of dry glutathione and 4 cc. of phosphate-citrate buffer (McIlvaine's series) were transferred to the lower part of a Thunberg tube. 1 cc. of 20 mg. per cent ascorbic acid oxidized by means of iodine in unbuffered aqueous solution was pipetted into the overhang. After evacuation with an oil pump the two solutions were mixed and the vessel then immersed in a water bath at 37.5°. At the end of 4 hours the volume of the solution was marked, the vacuum broken, 2 cc. of 10 per cent metaphosphoric acid added immediately after, and then aliquots of the solution titrated with dye in the usual manner. The glutathione present did not interfere with the end-point until its concentration was 300 mg. per cent. With this and higher concentrations of glutathione the end-points were uncertain. Parallel experiments with glutathione alone, at all the concentrations used, and dehydroascorbic acid alone were also carried out. The blanks in every case except with the two highest concentrations of glutathione were negligible.

We examined the possibility that this reaction between glutathione and dehydroascorbic acid was peculiar to solutions containing HI; *i.e.*, resulting from the oxidation of the ascorbic acid by iodine. This possibility was mentioned by Pfankuch in explanation of the discrepancy between his negative results and the positive results reported by Szent-Györgyi. Accordingly the experiment of Table X with 200 mg. per cent of glutathione was repeated with ascorbic acid oxidized in one case, by bromine (the oxidizing agent used by Pfankuch), and in another by oxygen catalyzed by copper. The figures show that somewhat higher yields were obtained with these oxidizing agents than with iodine.

We also investigated at this stage the effect, if any, of the composition of the buffer solution. Different concentrations of the same buffer salt mixture and none at all (in which the pH was maintained by addition of alkali), were tried. In four such solutions differing with respect to the amount of salt present the same degree of reduction was obtained with 200 mg. per cent of glutathione and an initial concentration of 4 mg. per cent of oxidized ascorbic acid.

We have concluded therefore that neither the oxidizing agent used in the oxidation of the ascorbic acid nor the composition of the buffer solution is significant in the reduction of dehydroascorbic acid by glutathione.



A much shorter time than 4 hours is required for the attainment of a nearly maximum reduction of added dehydroascorbic acid. Thus with 4 mg. per cent of dehydroascorbic acid and 200 mg. per cent of glutathione at pH 7.0 and 37.5°, *in vacuo*, the following degrees of reduction were found: in 5 minutes 52 per cent, in 10 minutes 73 per cent, in 15 minutes 80 per cent, in 120 minutes 86 per cent, and in 240 minutes 87 per cent. The failure to obtain 100 per cent reduction we ascribe mainly to the irreversible change in dehydroascorbic acid before it is reduced by the glutathione. At this pH and concentrations of glutathione and dehydroascorbic acid the interaction between the reductant and oxidant is very rapid; but a small fraction of the latter does undergo its irreversible

TABLE XI

*Effect of pH on Rate of Reduction of Dehydroascorbic Acid (10 Mg. Per Cent) by Glutathione (200 Mg. Per Cent) at 37.5°, in Vacuo*

| pH  | Reduction of ascorbic acid in |          |
|-----|-------------------------------|----------|
|     | ½ hr.                         | 20 hrs.  |
|     | per cent                      | per cent |
| 4   | <1                            | 13       |
| 5   | 6                             | 62       |
| 6   | 49                            | 79       |
| 6.5 | 71                            | 82       |
| 7.0 | 85                            | 86       |
| 8.0 | 91                            | 91       |

change. After this change it loses the property of reduction to the original form by glutathione in neutral solution (Table III).

*Effect of pH on Rate of Reduction of Dehydroascorbic Acid by Glutathione*—The higher the pH the faster is the rate of reduction of dehydroascorbic acid by glutathione. This rate increases faster with increasing pH than the rate of the irreversible change in dehydroascorbic acid. Hence the highest yields of the series were obtained at pH 8.0.

*Effect of Concentration of Dehydroascorbic Acid on Its Rate of Reduction by Glutathione*—The degree of reduction of dehydroascorbic acid at any given pH and temperature is dependent mainly on the concentration of glutathione.

Table XII shows that with 200 mg. per cent of glutathione

nearly the same percentage reduction was obtained in 15 to 30 minutes with concentrations of ascorbic acid varying from 2 to 50 mg. per cent. This relation together with the speed at which dehydroascorbic acid is reduced by glutathione suggests that there is some preliminary compound formation between it and glutathione.

TABLE XII

*Rate of Reduction of Different Concentrations of Dehydroascorbic Acid by 800 Mg. Per Cent of Glutathione at pH 7.0, and 37.5°, in Vacuo*

| Dehydro-<br>ascorbic acid | Reduction of added dehydroascorbic acid in different time intervals |          |          |          |          |
|---------------------------|---|----------|----------|----------|----------|
|                           | 5 min.  | 15 min.  | 30 min.  | 120 min. | 240 min. |
| mg. per cent              | per cent  | per cent | per cent | per cent | per cent |
| 2                         |   | 85       |          | 91       |          |
| 4                         | 66  | 76       | 86       | 85       | 87       |
| 10                        | 65  | 83       | 86       | 86       | 88       |
| 25                        | 57  | 84       | 81       |          |          |
| 50                        | 48  |          | 72       |          |          |
| 100                       | 32  |          | 57       |          |          |

TABLE XIII

*Rate of Reduction of Dehydroascorbic Acid in Vacuo and in Air with Different Concentrations of Glutathione and Ascorbic Acid in 15 Minutes, at pH 7.0, and 37.5°*

| Glutathione  | Per cent dehydroascorbic acid reduced |        |  |        |
|--------------|---------------------------------------|--------|--|--------|
|              | Initial concentration, 4 mg. per cent |        | Initial concentration, 10 mg. per cent |        |
|              | In vacuo                              | In air | In vacuo                               | In air |
| mg. per cent |                                       |        |  |        |
| 20           | 12                                    | 10     | 14                                     | 9      |
| 60           | 35                                    | 32     | 30                                     | 33     |
| 100          | 54                                    | 56     | 56                                     | 54     |
| 150          | 69                                    | 72     | 70                                     | 73     |
| 200          | 80                                    | 80     | 81                                     | 78     |

thione. The rate is much too rapid for a third order reaction; i.e., between 2 separate molecules of glutathione and 1 of dehydroascorbic acid. Bersin and his collaborators found some evidence of compound formation between glutathione and reduced ascorbic acid.

*Reduction of Dehydroascorbic Acid by Glutathione in Vacuo and*

*in Air*—Table XIII shows that the rate of reduction of dehydroascorbic acid by glutathione is as rapid in air as *in vacuo*. This holds true over a wide range of glutathione concentrations, and probably, though here only two concentrations have been tested, also over a wide range of dehydroascorbic acid concentrations.

TABLE XIV

*Recovery of Ascorbic Acid from Dehydroascorbic Acid (10 Mg. Per Cent) in Vacuo and in Air at 37.5° after Different Time Intervals in Solutions Containing Glutathione (200 Mg. Per Cent) and Different Metallic Impurities, and at Different Hydrogen Ion Concentrations*

| Variety of glutathione | pH | <i>In vacuo</i> or in air | Per cent of original dehydroascorbic acid recovered in reduced form after different time intervals |         |       |        |        |        |        |         |
|------------------------|----|---------------------------|--|---------|-------|--------|--------|--------|--------|---------|
|                        |    |                           | 0.25 hr.   | 0.5 hr. | 1 hr. | 2 hrs. | 4 hrs. | 5 hrs. | 7 hrs. | 20 hrs. |
| Crude                  | 7  | Air                       |  | 64      | 60    | 48     |        |        |        |         |
| "                      | 7  | Vacuum                    |  | 73      |       |        | 76     |        |        |         |
| "                      | 8  | Air                       |  | 81      | 66    | 11     |        |        |        |         |
| "                      | 8  | Vacuum                    |  | 85      |       |        | 90     |        |        |         |
| Cadmium                | 7  | Air                       |  | 54      | 48    | 31     |        |        |        |         |
| "                      | 7  | Vacuum                    |  | 63      |       |        | 65     |        |        |         |
| "                      | 8  | Air                       |  | 82      | 66    | 5      |        |        |        |         |
| "                      | 8  | Vacuum                    |  | 80      |       |        | 79     |        |        |         |
| Alcohol                | 6  | Air                       |  | 52      | 50    |        |        | 53     | 48     | 34      |
| " + Cu                 | 6  | "                         |  |         | 70*   |        |        | 23*    | 3      |         |
| "                      | 6  | Vacuum                    |  | 49      |       |        |        |        |        | 79      |
| "                      | 7  | Air                       | 78   | 85      | 75    | 69     | 53     |        |        | 0       |
| " + Cu                 | 7  | "                         |  | 101*    | 99*   | 90*    | 4*     |        |        |         |
| "                      | 7  | Vacuum                    | 82   | 85      | 86    |        | 86     |        |        | 86      |
| "                      | 8  | Air                       |  | 92      | 87    | 64     | 0      |        |        | 0       |
| " + Cu                 | 8  | "                         |  | 91*     | 108*  | 24*    | 0      |        |        | 0       |
| "                      | 8  | Vacuum                    | 91   | 91      |       |        |        | 91     |        | 91      |

Crude designates glutathione prepared and purified by the usual copper precipitation; cadmium is the crude material subjected to further purification by way of the cadmium salt; and alcohol the crude material precipitated from aqueous solution by alcohol.

\* Denotes that the end-point was fugitive and arbitrarily chosen. Only part of the titration value is to be attributed to ascorbic acid, the remainder probably to cysteine arising from the hydrolysis of the glutathione.

This can be observed only if the period of incubation is relatively short—15 minutes to  $\frac{1}{2}$  hour. In a longer time air oxidation of both the ascorbic acid formed and the glutathione, accompanied

by hydrolysis of the latter, supervenes (Table XIV). The figures show that the yields obtained in air are affected by metallic impurities present. The addition of copper (182 mm of  $\text{CuCl}_2$  per liter) accelerated, in air, the destruction of the ascorbic acid and the glutathione. The figures marked with an asterisk were those in which the behavior of the end-point in the dye titration indicated the presence of cysteine, arising from the hydrolysis of the glutathione. Titrations for glutathione indicated that in the air experiments a large fraction (50 per cent or more) had disappeared in 7 hours, and none remained after standing overnight.

Three preparations of glutathione were used in order to examine the effect of impurities. One preparation, designated as crude in Table XIV, was prepared and purified by the usual copper precipitation. Some of the crude material was purified further by way of the cadmium salt; it is designated as cadmium. A third alcohol product was prepared by precipitating the crude material from aqueous solution by alcohol. The crude material contained 91 per cent of the theoretical quantity of—SH groups, the other two preparations 97.5 per cent. Table XIV shows that impurities are less significant *in vacuo* than in air. The rates of reduction *in vacuo* with the three preparations were in the inverse order of their destruction in air. Judged by these two criteria the cadmium preparation contained the most impurity, the alcohol preparation the least. In all the other experiments recorded in this paper the alcohol preparation was used.<sup>1</sup>

We have compared the following twenty-nine substances with glutathione with respect to their ability to reduce dehydroascorbic acid: *dl*-alanine, *l*-asparagine, *l*-aspartic acid, *l*-cysteine, lithium formate, fructose, sodium fumarate, sodium glycerophosphate, galactose, glucose, *d*-glutamic acid, glycine, calcium hexosediphosphate, *l*-histidine, sodium *l*(+)-lactate, *l*-leucine, *d*-lysine, sodium *l*-malate, sodium maleate, *dl*-methionine, *d*-ornithine, *dl*-phenylalanine, *l*-proline, lithium pyruvate, *dl*-serine, sodium succinate, *l*-tryptophane, *l*-tyrosine, and *d*-valine. The experiments were carried out in air, at pH 7.0 and 37.5°. The initial concentration of dehydroascorbic acid was 10 mg. per cent, that of the other substances 0.01 M.

<sup>1</sup> We are indebted for these preparations of glutathione to Mr. S. W. Fox.

Except with cysteine no reduction was found with any of these substance in 15 minutes. In 60 minutes reduction equivalent to 3 per cent was found with lithium pyruvate, 2 per cent with calcium hexosediphosphate, and 1 per cent with sodium *l*-malate. The remainder gave less than 1 per cent or none.

Cysteine was conspicuous in this group as the only substance capable of reducing dehydroascorbic acid at a significant rate. Compared with glutathione, at the same molal concentration the amino acid was one-half as effective as the tripeptide. In other respects its action was similar. In the first 15 minutes the same degree of reduction was obtained in air and *in vacuo*. On longer incubation in air a lower yield was obtained on account of the oxidation of the cysteine and of the ascorbic acid formed.

Von Euler, Karrer, and Zehender (44) reported that dehydroascorbic acid may oxidize leucine, with the formation of ammonia and reducing substances. Similarly Heard and Welch (17) stated that the primary oxidation product of ascorbic acid is capable of oxidizing pyruvate and amino acids. Our observations indicate that none of the amino acids (except cysteine) nor pyruvate is capable of reducing dehydroascorbic acid at a significant rate. It would seem a safe generalization that only the —SH group is physiologically significant in this respect.

It is interesting that glucose, glucose dehydrogenase plus its coenzyme, glutathione, a hemochromagen, ascorbic acid, and oxygen constitute a complete respiratory system in which a metabolite is burned and oxygen used. The system is a simple one involving only one enzyme. All its components have been found in the tissues. It is probably this system which is responsible for the higher oxygen consumption observed by Quastel and Wheatley (41) when ascorbic acid is added to liver slices.

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# SUMMARY

1. Data are presented on the reversible potentials of the first and second oxidation stages of ascorbic acid.

2. These data and other experiments account for most of the previous discrepancies in the determination of the reversible oxidation-reduction potential of the first oxidation stage.

3. Proof is presented that only the first oxidation, ascorbic acid  $\rightleftharpoons$  dehydroascorbic acid, is physiologically reversible and significant in its antiscorbutic action.

4. An irreversible non-oxidative change in dehydroascorbic acid intervenes between the first and second oxidation stages. This irreversible change underlies the following phenomena: the negative drift in the oxidation-reduction potential measurements, the loss of reversibility of the first oxidation stage in the electrometric and colorimetric potential measurements, the increase in reducing power, the increase in strength of the dissociation constant of the ionizable hydrogen, the inability of  $H_2S$  in dilute acid solution and of glutathione in neutral and alkaline solutions to restore ascorbic acid from solutions of the oxidized vitamin, and the loss in antiscorbutic potency.

5. All of these manifestations of the irreversible change in dehydroascorbic acid exhibit the same dependence on the hydrogen ion concentration.

6. They are similarly independent of the presence of air or oxidizing agents (where air does not interfere with the reaction employed to show the irreversible change).

7. They are all similarly in accord that the change is an irreversible one; *i.e.*, that the two forms of the first oxidation product are not in equilibrium in dilute solution.

8. Ascorbic acid is oxidized very slowly in whole blood (human). It remains in the reduced state much longer in whole blood than in plasma.

9. The red blood corpuscles of beef, cat, dog, human, pig, rat, and sheep are nearly, if not absolutely, impermeable to added ascorbic acid.

10. There is no mechanism in human blood for reducing dehydroascorbic acid, or for retarding its irreversible change.

11. The antiscorbutic potency of dehydroascorbic acid is the

same as that of ascorbic acid, whether administered *per os* or subcutaneously. Yet *in vitro* at the pH and temperature of the tissues, dehydroascorbic acid quickly undergoes an irreversible change, whereby it loses its antiscorbutic potency.

12. This difference in its behavior *in vivo* and *in vitro* is the result of the rapid reduction of dehydroascorbic acid in the tissues. This reduction does not occur in the blood.

13. Evidence is presented that glutathione is probably the principal reducing agent here.

14. Some of the conditions are described affecting the rate of the reaction between glutathione and dehydroascorbic acid.

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## SULFHYDRYL OXIDATION-REDUCTION POTENTIALS DERIVED FROM THERMAL DATA

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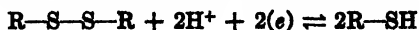
The isolation of glutathione by Hopkins in 1921 and his experiments suggesting that this compound is probably an intermediary in biological oxidations and reductions awakened an active interest in the biological significance of sulfhydryl compounds in general (1, 2). Among the fundamental data pertaining to these compounds are their oxidation-reduction potentials. Soon after the discovery of glutathione a number of attempts were made to measure these potentials by electrometric and colorimetric methods. In all except the most recent investigations the potential observed in aqueous solutions with noble metal electrodes was independent of the concentration of the oxidized form ( $R-S-S-R$ ) (3-5). The data fitted an empirical electrode equation of the form

$$E = E_0 - \frac{RT}{F} \text{pH} - \frac{RT}{nF} \ln (R-SH)$$

instead of an equation of similar form in which the last term is

$$\frac{RT}{nF} \ln \frac{(R-SH)^2}{R-S-S-R}$$

Accordingly these potentials are not referable to the reaction



whose characteristic potential was sought; and their quantitative significance will be obscure until the actual electrode reaction in each case is established.

Whatever be the explanation for this behavior of sulfhydryl compounds *in vitro*, knowledge of the value of the free energy change (potential) for the, at least hypothetically, reversible system  $R-S-S-R + 2H^+ + 2e \rightleftharpoons 2R-SH$  is important because glutathione and cysteine are oxidized and reduced *in vivo* (6-8), by enzymes in the presence of suitable substrates (9, 10), without enzymes by certain tissue extracts (11), and by ascorbic acid (12).

The solution of this problem was undertaken in this laboratory by application of thermal data in conjunction with the third law of thermodynamics. For this method the substances under investigation must be obtained in a high degree of purity; and a relatively large quantity of material, about 40 gm., is required. For these reasons the substances studied first were cysteine, cystine,  $\beta$ -thiolactic acid, and its disulfide oxidation product  $\beta,\beta'$ -dithiodilactic acid. The values obtained give some indication of the probable order of magnitude of the free energy change for the corresponding glutathione system. The preparation of oxidized and reduced glutathione in a sufficiently high degree of purity is in progress. When these preparations are completed, direct measurements on these substances will be made.

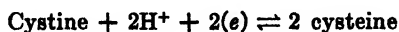
The great advantage offered by reliable thermal data in the computation of free energy changes, which may be expressed as equilibrium constants or oxidation-reduction potentials, is that the chemical reaction is precisely defined. In the case of equilibrium studies, especially where these involve electrode potential measurements, the actual reaction which occurs is often only guessed at. For these reasons the thermal data yield the final reference values.

Observations have been published recently from two different laboratories which leave little room for doubt that the potentials observed are those of thermodynamically reversible systems of which  $R-SH$  and  $R-S-S-R$  compounds are among the components. One group of workers, Ghosh *et al.* (13), employed an electrometric method; the other, Fruton and Clarke (14), a colorimetric method. The values obtained by these two methods for apparently the same system, *e.g.* cystine-cysteine, nevertheless are widely different and far beyond the probable experimental error. The thermal data presented below indicate that it is im-

TABLE I  
*Thermodynamic Data Pertaining to L-Cysteine, l-Cysteine,  $\beta$ -Thiolactic Acid, and  $\beta$ ,  $\beta'$ -Dithiodilactic Acid, at 25° and 37°*

|   | Standard state                         |   |                                     |  | In aqueous solution              |   |                              |
|---|--|---|-------------------------------------|--|----------------------------------|---|------------------------------|
|   | Entropy of formation, $\Delta S^\circ$ | Heat of combustion at constant pressure | Heat of formation, $\Delta H^\circ$ | Free energy of formation, $\Delta F^\circ$ | Solubility                       | $\frac{RT \ln N(\text{saturated})}{N(m=1)}$ | $\Delta F^\circ (1 M)$       |
| At 25°  |  |   |                                     |  |                                  |   |                              |
| L-Cysteine (solid).....                           | -152.3                                 | 532,200                                 | -127,880                            | calories per mole<br>-82,480               | mole per 1000 gm. water<br>0.229 | calories per mole<br>-870                   | calories per mole<br>-81,610 |
| l-Cysteine ".....                                 | -286.1                                 | 997,770                                 | -251,920                            | -166,630                                   | 0.000456                         | -4550                                       | -162,080                     |
| Thiolactic acid (liquid).....                     | -99.7                                  | 511,300                                 | -112,140                            | -82,420                                    | Miscible in all proportions      | +2390                                       | -84,810                      |
| $\beta, \beta'$ -Dithiodilactic acid (solid)..... | -212.0                                 | 946,520                                 | -232,150                            | -168,950                                   | 0.00639                          | -3050                                       | -65,900                      |
| At 37°  |  |   |                                     |  |                                  |   |                              |
| L-Cysteine.....                                   |  |   |                                     | -80,650                                    | 0.404                            | -550  | -80,110                      |
| l-Cysteine.....                                   |  |   |                                     | -163,200                                   | 0.000671                         | -4490                                       | -158,710                     |

probable that the chemical reaction whose potential was measured was in either case simply



*Free Energy of Formation in Standard State*—It is unnecessary here to present in detail the method of calculating equilibrium constants from thermal data. The general principles and the details of their application have been described in previous communications (15-17). The data necessary for calculating equilibria in aqueous solution are specific heats for the entropy values, heats of combustion for  $\Delta H$ , solubilities, ionization constants, and activity coefficients.

These data for the four compounds studied, except their ionization constants and activity coefficients, are given in Table I. The details of the preparation and purification of these compounds and of the thermal data are given elsewhere (18). The values for the heats and free energies of formation in the standard state given in Table I are slightly modified from the figures in our previous communication because of a change in the value of the heat of combustion of benzoic acid. The free energy values in the standard state at 37° for cysteine and cystine were calculated from the 25° values by means of the van't Hoff equation, which in its integrated form, on the assumption that  $\Delta H$  is constant over a short temperature interval, is

$$\frac{\Delta F_2}{T_2} - \frac{\Delta F_1}{T_1} = -\Delta H \left( \frac{T_2 - T_1}{T_1 T_2} \right) \quad (1)$$

*Free Energies of Formation of Neutral Forms in Aqueous Solution*—From this point on the calculation of the free energy changes in solutions at all hydrogen ion concentrations becomes complicated. In order to keep the direction clear we shall anticipate data on the ionization constants in Table III to obtain the values for the free energies of formation of the neutral forms in solution.

The free energy of formation in aqueous solution is given by the equation

$$\Delta F(1 \text{ M}) = \Delta F^\circ - RT \ln \frac{N(\text{saturated solution})}{N(m = 1)} \quad (2)$$

where  $N$  (saturated solution) is the mole fraction in the saturated solution, and  $N$  ( $m = 1$ ) is the mole fraction when 1 mole of the substance is dissolved in 1000 gm. of water. Mole fraction is defined by the equation  $m_1/(m_1 + m_2)$  where  $m_1$  and  $m_2$  are the numbers of moles of solute and solvent respectively. In the cases of the two thio acids the complication is encountered that a fraction of the material in the saturated solution is in the ionized form. The treatment of this complication is discussed below.

The values for the solubility of *l*-cystine in Table I are taken from the data of Dalton and Schmidt (19). The solubilities of the two remaining compounds were determined by means of a technique similar to that employed by these authors. Solubility tubes were shaken in an air bath at the temperature indicated for 48 to 72 hours, after which the solution was filtered at the temperature of the bath. The quantity of solid in a weighed quantity of solution was determined by evaporating the solution to dryness at 85–90°. Preliminary experiments showed that the two substances studied could be recovered quantitatively from their solutions by this method. In the case of *l*-cysteine the tubes containing the amino acid were half filled with freshly boiled water and the air was displaced by hydrogen to prevent oxidation during the period of shaking. The figures given in Table I are averages of a number of separate experiments in which the extremes differed by less than 6 per cent. This uncertainty is negligible in the calculation of the free energy of formation of the substance in solution.

In the saturated solutions all the substances undergo some ionization. In order to compute the free energy of formation in solution of any particular form this must be taken into account. The details of this calculation for cysteine are presented to illustrate the general method applicable to weak acids, amino acids, and weak bases, whether mono- or polyvalent. Proceeding from highly acid to highly alkaline solutions the ionic forms of cysteine will be designated as  $R^+$ ,  $R^\pm$ ,  $R^-$ , and  $R^{--}$ . In the saturated solution



The equilibrium expressions for the various forms are

$$\frac{(R^\pm)(H^+)}{(R^+)} = K_1; \quad \frac{(R^-)(H^+)}{(R^\pm)} = K_2; \quad \frac{(R^{--})(H^+)}{(R^-)} = K_3$$

If the total concentration of cysteine in all forms is designated as  $C$ , from the above relations,

$$C = (R^{\pm}) \left( \frac{(H^+)^2 + K_1(H^+)^2 + K_1K_2(H^+) + K_1K_2K_3}{K_1(H^+)^2} \right) \quad (4)$$

From Equation 3 and the equilibrium relations

$$\begin{aligned} (H^+) &= \frac{K_w}{(H^+)} + (R^{\pm}) \left( \frac{-(H^+)^2 + K_1K_2(H^+) + 2K_1K_2K_3}{K_1(H^+)^2} \right) \\ &= \frac{K_w}{(H^+)} + C \left( \frac{-(H^+)^2 + K_1K_2(H^+) + 2K_1K_2K_3}{(H^+)^2 + K_1(H^+)^2 + K_1K_2(H^+) + K_1K_2K_3} \right) \end{aligned} \quad (5)$$

At  $25^\circ$ ,  $C$  is  $2.29 \times 10^{-1}$ ; and from Table III the values of  $K_1$ ,  $K_2$ , and  $K_3$  are  $1.95 \times 10^{-2}$ ,  $4.57 \times 10^{-9}$ , and  $1.66 \times 10^{-11}$  respectively.

Equation 5 is simplified by substituting the limits of the possible values of  $(H^+)$  and eliminating the negligible terms. Through the range from pH 4 to 8, Equation 3 becomes

$$(H^+) = \frac{K_w}{(H^+)} + C \left( \frac{K_1K_2 - (H^+)^2}{K_1K_2 + K_1(H^+)^2} \right) \quad (6)$$

From Equation 6 it is seen that  $(H^+)^2$  must be less than  $K_1K_2$ . With  $10^{-5.5}$  and  $10^{-6.0}$  for  $(H^+)$  the drift of the disagreement between the left- and right-hand sides of Equation 4 shows that the value of  $(H^+)$  which will satisfy this equation lies between  $10^{-5.0}$  and  $10^{-5.5}$ . In this case  $K_1K_2$  in the denominator is negligible compared with  $K_1(H^+)^2$ . With this elimination the value of  $(H^+)$  becomes  $10^{-5.04}$ . On substituting this value into Equation 4 it is found that practically all of the amino acid in the saturated aqueous solution is in the zwitter ionic form. The same is true for cystine.

$\beta$ -Thiolactic acid is miscible with water in all proportions. The hydrogen ion concentration and the degree of ionization will depend upon the concentration of the acid. However, at 1 m less than 1 per cent is ionized. For the calculation of the free energy of solution we may assume that all the acid, both when the concentration of water is 0 and when the acid is at 1 m concentration in water, is in the neutral form. The calculated pH of the saturated aqueous solution of  $\beta, \beta'$ -dithiodilactic acid is approximately 3.16, corresponding to ionization of 11 per cent of

the acid. In computing the free energy of solution the mole fraction of the saturated solution  $N_s$  was multiplied by the fraction of the material which is present in the neutral form  $\alpha$ , here 0.89. The term for the free energy of the solution becomes

$$RT \ln \frac{N(\text{saturated solution})}{N(m=1)} \alpha$$

In the cases of the three other substances the value of  $\alpha$  is 1. In computing the free energy of solution of substances miscible with water in all proportions, as in the case here of  $\beta$ -thiolactic acid, the term for  $N(\text{saturated solution}) = 1$ .

Table I gives the values of  $\Delta F(1 \text{ M})$  at 25° for the four substances, and at 37° for cysteine and cystine.

*Ionization Constants and Activity Coefficients*—In order to express the free energy changes at all hydrogen ion concentrations in a useful form, *i.e.* in terms of the total concentrations of oxidant and reductant, the ionization constants of these substances must be known. Where they may take the form of polyvalent ions, it is better to attempt an approximate determination of the thermodynamic ionization constants rather than to leave them in the form of titration constants. Otherwise systematic differences may appear between the observed and theoretical oxidation-reduction potentials at different hydrogen ion concentrations (15, 16), because in the usual experimental solutions, *e.g.* where buffers are used, the ionic strength may be quite high, and will be different at different hydrogen ion concentrations. As a result the corrections for activity coefficients are large, they may be different for oxidant and reductant, and will vary at different pH values of the experimental solutions.

Accordingly we have attempted estimation of the thermodynamic ionization constants of the four substances studied here. These estimations are based on electrometric titrations, except in the case of cystine. For this substance solubility data were used. In the electrometric titrations the hydrogen electrode against a saturated calomel cell was employed for cysteine and  $\beta$ -thiolactic acid, the glass electrode for  $\beta, \beta'$ -dithiodilactic acid. With cysteine one titration was made with platinized asbestos suspended in the solution, and with  $\beta$ -thiolactic acid colloidal platinum, to minimize oxidation of the sulphydryl group during the titration.



The values obtained were the same as when this precautionary measure was omitted.

In every case complications arising from overlapping ionization of these polyvalent compounds are encountered. The procedure with cysteine will again be chosen to illustrate the general method employed in deriving the individual titration and thermodynamic ionization constants. The calculations are long and laborious, particularly for the ionization in alkaline solutions. In order to facilitate following the development a few examples of actual data are given in Table II.

TABLE  
Calculation of Ionization Constants of L-Cysteine

| NaOH added | Volume of solution | Concentration of added NaOH | pH    | $A_{H^+}$               | Total concentration of cysteine in all forms | Approximate concentrations for purpose of estimating ionic strength, $\mu$ , of |                   |                   | Ionic strength, $\mu$ | $K_a \times \gamma_{H^+} / \gamma_{\pm} \times 10^{-14}$ | $\gamma_{H^+} = \gamma_{\pm}$ |   |
|------------|--------------------|-----------------------------|-------|-------------------------|--|---|-------------------|-------------------|-----------------------|--|-------------------------------|---|
| (1)        | (2)                | (3)                         | (4)   | (5)                     | (6)  | (R <sup>±</sup> )   | (R <sup>-</sup> ) | (R <sup>=</sup> ) | (10)                  | (11)   | (12)                          | " |
| cc.        | cc.                | mole per l.                 |       | mole per l.             | mole per l.                                  | mole per l.   | mole per l.       | mole per l.       |                       |  |                               |   |
| 2 00       | 27 00              | 0 005739                    | 7 35  | $4.487 \times 10^{-3}$  | 0 0500                                       | 0 0435  | 0 00650           | 0                 | 0 0067                | 1 106  | 0 92                          | 2 |
| 5 00       | 30 00              | 0 01262                     | 7 395 | $1.274 \times 10^{-2}$  | 0 0450                                       | 0 02925   | 0 01575           | 0                 | 0 0129                | 1 127  | 0 895                         | 8 |
| 15 04      | 40 04              | 0 02912                     | 9 03  | $9.376 \times 10^{-10}$ | 0 03375                                      | 0 004388  | 0 02335           | 0 00101           | 0 0298                | 1 180  | 0 85                          | 1 |
| 19 04      | 44 04              | 0 03353                     | 9 70  | $1.977 \times 10^{-10}$ | 0 03068                                      | 0 000920  | 0 0255            | 0 00491           | 0 0367                | 1 199  | 0 84                          | 6 |
| 25 06      | 50 06              | 0 03876                     | 10 41 | $3.682 \times 10^{-11}$ | 0 02696                                      | 0 000135  | 0 0140            | 0 0129            | 0 0480                | 1 224  | 0 83                          | 3 |
| 31 10      | 56 1               | 0 04295                     | 10 95 | $1.117 \times 10^{-11}$ | 0 02406                                      |   | 0 00329           | 0 0188            | 0 0556                | 1 238  | 0 83                          | 1 |

0.1635 gm. of cysteine dissolved in 25 cc. of water; NaOH = 0.07748 N, 25°.  $pK_2 = pH$   
 $-\log \gamma_{\pm} = (0.5 \times 3.3\sqrt{\mu}) / (1 + 0.33\sqrt{\mu})$

The ionization relations of cysteine may be written as follows:

$$\frac{(R^{\pm})(H^+)}{(R^+) \gamma_{\pm}} = K_1; \quad \frac{(R^-)(\gamma_{\pm})(H^+)}{R^{\pm}} = K_2; \quad \frac{(R^-)(\gamma_{\pm})(H^+)}{(R^-)(\gamma_{\pm})} = K_3$$

$R^+$  is the form in acid solution in which the ionization of the carboxyl group is suppressed,  $R^{\pm}$  the zwitter ion,  $R^-$  the form in which presumably the ionization of the amino group is suppressed, and  $R^-$  the form in which the sulfhydryl group is ionized.  $\gamma_{\pm}$ ,  $\gamma_{-}$ , and  $\gamma_{\pm}$  are the corresponding activity coefficients.

In all these computations the activity coefficient of the zwitter ion or neutral molecule in the case of the thio acids was taken as unity. The observations of Harned and Owen (20) on acetic acid

and glycine (21) and of Pfeiffer and Würzler (22) and of Cohn (23) on the solubility of amino acids in salt solutions indicate that at the concentration of amino acids and salts in our solutions little error was incurred by this assumption.

The first dissociation constant  $pK_1$  is given by the expression

$$pK_1 = pH + \log(R^+) + \log \gamma_+ - \log(R^\pm)$$

( $R^+$ ) was taken as the difference between the concentration of hydrochloric acid added and the concentration of hydrogen ions in the solution. The latter quantity is the antilog of the nega-

## II

### on Alkaline Side of Neutrality; $pK_2$ and $pK_3$

| $M_{OH} =$<br>(11)<br>(5) | $\frac{N}{\text{Bound (Na)}} =$<br>(3) - (15) | $\frac{N}{(R^\pm) = (6) - (17) - (21)}$ | $-\log(R^\pm)$ | $(R^-)$     | $-\log(R^-)$ | $\frac{N}{-\log \gamma_-}$<br>(12) | $\frac{N}{pK_2 = (4) + (16) - (18)}$ | $\frac{N}{(R^-) = (9) - (16) - (17)}$ | $\frac{N}{-\log(R^-) = (21) - \log(21)}$ | $-\log \gamma_-$ | $\frac{N}{pK_3 = (4) + (20) - (16) - (18)}$ |
|---------------------------|---|---|----------------|-------------|--------------|------------------------------------|--------------------------------------|---------------------------------------|--|------------------|---|
| (13)                      | (14)  | (15)                                    | (16)           | (17)        | (18)         | (19)                               | (20)                                 | (21)                                  | (22)                                     | (23)             | (24)  |
| mole per l.               | mole per l.                                   | mole per l.                             |                | mole per l. |              |                                    |                                      | mole per l.                           |  |                  |   |
| $476 \times 10^{-7}$      | 0 006738                                      | 0 04426                                 | 1 354          | 0 006738    | 2 241        | 0 086                              | 8 27                                 |                                       |  |                  |   |
| $148 \times 10^{-7}$      | 0 01291                                       | 0 03209                                 | 1 494          | 0 01291     | 1 889        | 0 048                              | 8 34                                 |                                       |  |                  |   |
| $358 \times 10^{-8}$      | 0 02912                                       | 0 00645                                 | 2 264          | 0 02747     | 1 561        | 0 069                              | 8 39                                 |                                       |  |                  |   |
| $368 \times 10^{-8}$      | 0 03947                                       |   |                | 0 02654     | 1 576        | 0 074                              |                                      | 0 00371                               | 2 431                                    | 0 398            | 10 78                                       |
| $158 \times 10^{-8}$      | 0 03944                                       |   |                | 0 01541     | 1 812        | 0 079                              |                                      | 0 01149                               | 1 940                                    | 0 337            | 10 80                                       |
| $198 \times 10^{-8}$      | 0 04175                                       |   |                | 0 00628     | 2 202        | 0 083                              |                                      | 0 01778                               | 1 750                                    | 0 361            | 10 78                                       |

$$[-\log(R^-) - \log \gamma_- + \log(R^\pm)]. \quad pK_3 = pH - \log(R^-) - \log \gamma_- - \log(R^-) - \log \gamma_-.$$

tive of the pH observed,  $A_H$ , divided by the activity coefficient of the hydrogen ion in the given solution.

The hydrogen ion activity coefficients at different ionic strengths were calculated from the data of Harned and Hamer (24) on the activity of HCl in KCl solutions, and of Spencer (25) of KCl in aqueous solutions at 25° by means of the following equations.

$$\gamma_{HCl}^2 = \gamma_{H^+} \times \gamma_{Cl^-}$$

$$\gamma_{KCl}^2 = \gamma_{K^+} \times \gamma_{Cl^-}$$

The assumption was made that in these dilute solutions  $\gamma_{K^+} = \gamma_{Cl^-}$ .

Then  $\gamma_{KCl} = \gamma_{Cl^-}$  and  $\gamma_{HCl}^2 = \gamma_{H^+} \times \gamma_{KCl}$ ;  $\gamma_{H^+} = \gamma_{HCl}^2 / \gamma_{KCl}$ .

The values so obtained were preferred to those given by the empirical equations of Larsson and Adell (26) and of Bjerrum and Unmack (27).

The difference between the total concentration of amino acid present and the concentration of bound acid is ( $R^\pm$ ), the concentration of the zwitter ion.

The activity coefficients of the monovalent cation and anion of the amino acids or thio acids were taken to be the same as those of the hydrogen ion.

In the calculation of the basic dissociation constants it was assumed that the salts of the amino acids and thio acids were completely dissociated. The amount of salt present was calculated from the bound base which was obtained by subtracting the concentration of hydroxyl ions,  $M_{OH^-}$ , from the concentration of NaOH added.  $M_{OH^-}$  was computed from the observed pH as follows:

$$K_w = \frac{\gamma_{H^+} \times \gamma_{OH^-}}{A_{H_2O}} \times M_{H^+} \times M_{OH^-} = \gamma_w \times M_{H^+} \times M_{OH^-}$$

$$M_H = \frac{A_{H^+}}{\gamma_{H^+}}; \quad M_{OH^-} = \frac{K_w}{\gamma_w} \times \frac{\gamma_{H^+}}{A_{H^+}} \quad (7)$$

$K_w$  and  $\gamma_w$  at different ionic strengths were determined by Harned and Hamer (24);  $A_{H^+}$  is the antilog of the negative of the pH observed; and  $\gamma_{H^+}$  was calculated as described above.  $(B) - M_{OH^-}$ , where  $(B)$  is the concentration of added base, is the bound base.

The bound base is divided between  $R^-$  and  $R^-$ . To determine the partition the approximate concentration of  $R^-$  was computed from the titration constants,  $K'_2$  and  $K'_3$ , taken by inspection from the titration curve, for the first part of the alkaline titration where  $(R^-)$  is very low. The bound base minus  $2(R^-)$  gives  $(R^-)$ , the concentration of  $R^-$ . The total concentration of amino acid minus  $(R^-)$  and  $(R^-)$  gives  $(R^\pm)$ , the concentration of zwitter ion. At the highly alkaline end of the titration curve  $(R^\pm)$  was calculated by means of  $K'_2$  and  $K'_3$ . The calculated quantity at each pH was subtracted from the total concentration of amino acid present. The difference between the total bound

base and this quantity divided by 2 gives  $(R^-)$ . The total bound base minus  $(R^\pm)$  minus  $2(R^-)$  gives  $(R^-)$ .

The following are the equations for computing  $(R^\pm)$  and  $(R^-)$

$$R^\pm = C \left( \frac{(H^+)^2}{(H^+)^2 + K_1(H^+) + K_1K_2} \right)$$

and

$$R^- = C \left( \frac{K_2K_3}{(H^+)^2 + K_1(H^+) + K_1K_2} \right)$$

where  $C$  is the total concentration of amino acid.

The titration constants were also used to compute the approximate ionic strength at each stage in the titration in order to obtain the activity coefficients of the different ions. For the activity coefficients of the divalent ions the following modified Debye-Hückel equation was employed.

$$-\log \gamma_{\pm} = \frac{(0.5)(3.3) \sqrt{\mu}}{1 + 0.33 \sqrt{\mu}} \quad (8)$$

3.3 instead of 4 was used in the numerator to allow for the distance between the charges in the divalent ion (28). Similarly, in assessing the contribution of the divalent ion to the ionic strength  $3.3(R^-)$  instead of  $4(R^-)$  was taken.

With these values for  $(R^\pm)$ ,  $(R^-)$ , and  $(R^-)$ , and their corresponding activity coefficients tentative values for  $K_2$  and  $K_3$  were obtained from the beginning and end of the titration curve. These values and correspondingly adjusted activity coefficients were used for recalculation of  $(R^-)$  and  $(R^\pm)$  at the initial and terminal stages of the titration. By this process of successive approximation values of  $(R^\pm)$ ,  $(R^-)$ , and  $(R^-)$  were obtained for final values of  $K_2$  and  $K_3$ .

The above treatment of the titration data did not give a constant value for  $pK_2$ . The value obtained increased with increasing pH. At pH 7.0  $pK_2$  found was 8.22, at pH 8.0, 8.33, and at pH 9.0, 8.37. This drift was independent of the ionic strength, identical values being obtained at the same pH over 10-fold variation in ionic strength. The excellent agreement of the  $pK_2$  values over a wide range of ionic strengths (Table III) also shows

TABLE III

*Ionization Constants at 25° of l-Cysteine, l-Cystine,  $\beta$ -Thiolactic Acid, and  $\beta,\beta'$ -Dithiodilactic Acid*

| Concentration of substance  | Ionic strength, $\mu$ | Activity coefficients used |                                 |                                 |              | Ionization constants |        |        |        |
|---|-----------------------|----------------------------|---------------------------------|---------------------------------|--------------|----------------------|--------|--------|--------|
|   |                       | $\gamma_{++}$              | $\gamma_{\pm+}$ or $\gamma_{+}$ | $\gamma_{\pm-}$ or $\gamma_{-}$ | $\gamma_{=}$ | $pK_1$               | $pK_2$ | $pK_3$ | $pK_4$ |
| <i>l-Cysteine</i>   |                       |                            |                                 |                                 |              |                      |        |        |        |
| mole per l.   |                       |                            |                                 |                                 |              |                      |        |        |        |
| 0.015   | 0.034                 |                            | 0.85                            |                                 |              | 1.70                 |        |        |        |
| 0.028   | 0.175                 |                            | 0.77                            |                                 |              | 1.71                 |        |        |        |
| 0.042   | 0.017                 |                            |                                 | 0.88                            |              |                      | 8.34   |        |        |
| 0.031   | 0.182                 |                            |                                 | 0.77                            |              |                      | 8.33   |        |        |
| 0.028   | 0.045                 |                            |                                 | 0.835                           | 0.47         |                      |        | 10.78  |        |
| 0.019   | 0.158                 |                            |                                 | 0.77                            | 0.26         |                      |        | 10.78  |        |
| Average value taken.....  |                       |                            |                                 |                                 |              | 1.71                 | 8.33   | 10.78  |        |
| Titration constants, $pK'$ , at 30°, concentration 0.02 M, of Cannan and Knight (29)..... |                       |                            |                                 |                                 |              | 1.96                 | 8.18   | 10.28  |        |
| <i>l-Cystine</i>  |                       |                            |                                 |                                 |              |                      |        |        |        |
| 0.00099   | 0.01                  | 0.69                       | 0.91                            |                                 |              | 0.96                 | 2.08   |        |        |
| 0.0019  | 0.02                  | 0.60                       | 0.88                            |                                 |              | 1.08                 | 2.03   |        |        |
| 0.014   | 0.11                  | 0.32                       | 0.80                            |                                 |              | 1.08                 | 2.05   |        |        |
| 0.0022  | 0.005                 |                            |                                 | 0.92                            |              |                      |        | 8.00   |        |
| 0.00099   | 0.10                  |                            |                                 | 0.80                            |              |                      |        | 8.01   |        |
| 0.0036  | 0.01                  |                            |                                 | 0.91                            | 0.69         |                      |        | 7.99   | 10.26  |
| 0.0069  | 0.02                  |                            |                                 | 0.88                            | 0.60         |                      |        | 7.96   | 10.23  |
| Average value taken.....  |                       |                            |                                 |                                 |              | 1.04                 | 2.05   | 8.00   | 10.25  |
| Titration constants, $pK'$ , at 25°, of Sano (30).....                                    |                       |                            |                                 |                                 |              | 1.60                 | 2.21   | 7.86   | 9.85   |
| Titration constants, $pK'$ , at 30°, concentration 0.02 M, of Cannan and Knight.....      |                       |                            |                                 |                                 |              | <1.0                 | 1.7    | 7.48   | 9.02   |
| <i><math>\beta</math>-Thiolactic acid</i>   |                       |                            |                                 |                                 |              |                      |        |        |        |
| 0.047   | 0.028                 |                            | 0.86                            | 0.825                           |              | 4.32                 |        |        |        |
| 0.029   | 0.059                 |                            |                                 |                                 |              |                      | 10.47  |        |        |
| <i><math>\beta,\beta'</math>-Dithiodilactic acid</i>                                      |                       |                            |                                 |                                 |              |                      |        |        |        |
| 0.0030  | 0.0046                | 0.78                       |                                 |                                 |              |                      | 4.99   |        |        |
| 0.0031  | 0.0056                | 0.91                       |                                 |                                 |              | 4.08                 |        |        |        |

that the drift cannot be ascribed to the use of incorrect activity coefficients. Further, at these low hydroxyl ion concentrations assumptions regarding the activity coefficient of the hydroxyl ion, or of the divalent anion, are negligible. The only reasonable explanation which suggests itself to us is association of the zwitter ion, increasing with increasing pH. If this explanation be correct the curve of  $pK_2$  against pH indicates that the true value is in the neighborhood of 8.1. The  $pK_2$  value chosen, 8.33, is that obtained in the mid-region of the titration curve of this group, between pH 8.0 and 8.4. The only titration data in the literature reported in sufficient detail to permit recalculation of the dissociation constants with suitable activity coefficients, those of Tague (31) for glycine and phenylalanine, show no evidence of such a drift in the ionization of the amino group as we have observed with cysteine. The data reported by Cannan and Knight (29) for cysteine at 0.1 M concentration at 30° are insufficient for the estimation of whether this drift was present in their titrations or not.

In the case of  $\beta$ -thiolactic acid the calculations are simpler because there are only two ionizable groups with widely separated constants.

In  $\beta,\beta'$ -dithiodilactic acid there are two overlapping dissociation constants in the acid region. The same procedure as in the case of the overlapping basic dissociation constants of cysteine was employed, the concentration of bound acid being used instead of bound base.

The solubility data of Sano (30) were used for the calculation of the four constants of cystine, each pair overlapping. It was assumed that over the whole range from pH 0.83 to 9.58 the concentration of the zwitter ion was the same and equal to the minimum solubility, 0.000457 mole per liter observed by Sano. It is implied in this assumption that the activity coefficient of the zwitter ion is unity throughout. If this minimum solubility, *i.e.*  $(R^{\pm\pm})$ , is represented by  $\lambda$ , the four equilibrium expressions are

$$\frac{\gamma_{\pm-}(R^{\pm-})(H^+)}{\gamma_{++}(R^{++})} = K_1; \quad \frac{\lambda(H^+)}{\gamma_{\pm+}(R^{\pm+})} = K_2; \quad \frac{\gamma_{\pm-}(R^{\pm-})}{\lambda} = K_3;$$

$$\frac{\gamma_{-}(R^{-})(H^+)}{\gamma_{\pm-}(R^{\pm-})} = K_4$$

where  $(H^+)$  represents the hydrogen ion activity. In acid solutions the concentration of the divalent cation,  $(R^{++})$ , is given by the expression  $\lambda(H^+)^2/K_1K_2 \gamma_{++}$  and in alkaline solutions the concentration of the divalent anion  $R^-$  by  $\lambda K_3K_4/(H^+)^2 \gamma_-$ .

Sano used a series of buffers for his solubility measurements at different hydrogen ion concentrations, with the result that the ionic strengths ranged, in the acid series, from 0.01 to 0.35, and, in the alkaline series, from 0.005 to 0.115. The calculated activity coefficients of the divalent ions varied accordingly from 0.69 to 0.15 in the acid range and from 0.92 to 0.28 in the alkaline range. For this reason the equilibrium constants involving the divalent

TABLE IV

*Calculated and Observed Solubility of Cystine at Different Hydrogen Ion Concentrations, in Buffer Solutions at 25°*

| pH   | Ionic strength, $\mu$ | Observed by<br>Sano (30) | Calculated from ionization constants |  |
|------|-----------------------|--------------------------|--------------------------------------|--|
|      |                       |                          | Of Sano                              | In Table III and<br>activity<br>coefficients |
|      |                       | <i>mole per l.</i>       | <i>mole per l.</i>                   | <i>mole per l.</i>                           |
| 7.52 | 0.10                  | 0.000689                 | 0.000664                             | 0.000645                                     |
| 7.98 | 0.10                  | 0.000985                 | 0.00107                              | 0.000994                                     |
| 8.55 | 0.005                 | 0.00222                  | 0.00280                              | 0.00226                                      |
| 8.78 | 0.01                  | 0.00363                  | 0.00457                              | 0.00363                                      |
| 9.05 | 0.02                  | 0.00687                  | 0.00864                              | 0.00684                                      |
| 9.85 | 0.115                 | 0.0521                   | 0.0898                               | 0.00473                                      |

cation and anion,  $K_1$  and  $K_4$ , are less trustworthy than  $K_2$  and  $K_3$ .

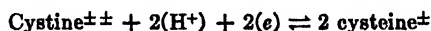
The four ionization constants were estimated by successive approximation, as in the case of the overlapping ionizing groups of cysteine. The same assumptions were made regarding activity coefficients. The differences between the pK values in Table III for cystine and those deduced by Sano from the same data are mostly to be ascribed to our introduction of activity coefficients, and to some extent to different weighting of the individual values. Table IV shows that a closer agreement is thus obtained between observed and calculated solubility at different hydrogen ion concentrations than with the constants given by Sano. These figures are presented because the interpretation given certain experimentally observed potentials for the cystine  $\rightleftharpoons$  cysteine

system, discussed below, depends on the accuracy of the calculated solubility of cystine in the experimental solutions.

In Table III are collected the values for the ionization constants obtained at the extremes of ionic strengths. The averages given are of a considerably larger number of determinations than those shown. The titration constants of Cannan and Knight and of Sano are given for comparison. The agreement in the calculated ionization constants even where there were large differences in activity coefficients shows that the values used for these are nearly correct or that the errors are compensatory.

*Oxidation-Reduction Potentials of Cystine  $\rightleftharpoons$  Cysteine*—With these data we can now proceed to the calculation of the oxidation-reduction potentials at different hydrogen ion concentrations of cystine  $\rightleftharpoons$  cysteine and of the  $\beta, \beta'$ -dithiodilactic acid  $\rightleftharpoons$  thiolactic acid systems. In both systems the reaction mechanisms postulated are between the neutral molecules. The calculated potential at any given hydrogen ion concentration is independent of the mechanism postulated. Our method of developing electrode potential equations to be used in conjunction with thermal data has been described in detail in previous communications (15, 17). We shall present here only the final equations.

For the postulated reaction



the electrode potential equation is

$$E_{\text{obs.}} = \bar{E} - \frac{RT}{2f} \ln \frac{(\text{R-SH})^2}{(\text{R-S-S-R})} - \frac{RT}{2f} \ln \frac{(K_1^{\text{SH}})^2}{K_1^{\text{SS}} K_2^{\text{SS}}} \\ \frac{RT}{2f} \ln \frac{(\text{H}^+)^4 + K_1^{\text{SS}}(\text{H}^+)^3 + K_1^{\text{SS}} K_2^{\text{SS}}(\text{H}^+)^2 + K_1^{\text{SS}} K_2^{\text{SS}} K_3^{\text{SS}}(\text{H}^+) + K_1^{\text{SS}} K_2^{\text{SS}} K_3^{\text{SS}} K_4^{\text{SS}}}{((\text{H}^+)^2 + K_1^{\text{SH}}(\text{H}^+)^2 + K_1^{\text{SH}} K_2^{\text{SH}}(\text{H}^+) + K_1^{\text{SH}} K_2^{\text{SH}} K_3^{\text{SH}})^2} \quad (9)$$

$\bar{E}$  is the molal electrode potential;  $R$ ,  $T$ , and  $f$  have their usual significance.  $(\text{R-SH})$  and  $(\text{R-S-S-R})$  are the concentrations of total cysteine and cystine respectively, *i.e.* the sum in all forms.  $K_1^{\text{SH}}$ ,  $K_2^{\text{SH}}$ ,  $K_3^{\text{SH}}$ ,  $K_4^{\text{SH}}$  are the ionization constants of cystine,  $K_1^{\text{SS}}$ ,  $K_2^{\text{SS}}$ ,  $K_3^{\text{SS}}$ ,  $K_4^{\text{SS}}$  the ionization constants of cysteine.

A more rigorous form of Equation 9 would include activity coefficients. We have computed potentials between pH 3 and



pH 9, with ionic strengths varying from 0 to 0.2, and have found that the difference, when activity coefficients are taken into account, is negligible. The activity coefficients used were those given in Tables II and IV.

The usual term,  $RT/f \times 2.303 \text{ pH}$ , cancels out in Equation 9. The dependence of the potential on the pH is implicit in the last term. The equation takes this form because cystine contains one more ionizable group than cysteine.

It is customary to tabulate electrode potentials of organic oxidants and reductants as  $E'_0$  values; i.e., the potentials at stipulated pH values when the concentrations of total oxidant and reductant are equal so that the corresponding log term in the electrode equation becomes 0. This mathematical convenience is not obtained here when the final concentrations of cystine and cysteine are equal. Accordingly, in Table V a series of potentials is given, in which the concentrations of cystine and cysteine are such that  $\log (R-SH)^2/(R-S-S-R)$  is 0. This reference potential is designated as  $E''_0$  to distinguish it from the commonly used  $E'_0$ .

In discussion of the reversibility of the cystine-cysteine system it has not been generally realized that even in those conditions where the system is reversible the potential will vary for the same ratio of cysteine to cystine with their actual concentrations. This is a result of the squaring of the cysteine concentration, while the cystine concentration is taken only to the first power. On theoretical grounds, therefore, we may expect more negative potentials, or the reduction of progressively more negative dyes, with increasing concentrations of cysteine. This, in itself, is not evidence of the irreversibility of the system under observation.

The value of  $\bar{E}$  in Equation 9 from the data in Table I is

$$\frac{-162,080 - 2(-81,610)}{2 \times 23,074} = +0.025 \text{ volt}$$

The value of the other constant term

$$- \frac{RT}{2f} \ln \frac{(K_1^{SH})^2}{K_1^{SS} K_2^{SS}}$$

will depend on the ionization constants chosen. We have recalculated Cannan and Knight's data on the dissociation of cystine and cysteine with the same assumptions regarding activity coefficients

TABLE V

*Oxidation-Reduction Potentials for Cystine  $\rightleftharpoons$  Cysteine, and  $\beta,\beta'$ -Dithiodilactic Acid  $\rightleftharpoons$   $\beta$ -Thiolactic Acid Systems, at 25° and 37°*

*Relation between  $E''_0$  (Potential When  $\frac{(R-SH)^2}{R-S-S-R} = 1$ ) and pH*

The values are measured in volts.

| pH | Cystine-cysteine |          |          |          | Dithiodilactic acid-thiolactic acid, 25° |
|----|------------------|----------|----------|----------|--|
|    | 25°              |          | 37°      |          |  |
|    | Series 1         | Series 2 | Series 1 | Series 2 |  |
| 0  | +0.034           | +0.059   | +0.041   | +0.068   | +0.081                                   |
| 1  | -0.028           | -0.007   | -0.024   | -0.001   | +0.022                                   |
| 2  | -0.093           | -0.083   | -0.092   | -0.080   | -0.037                                   |
| 3  | -0.152           | -0.156   | -0.154   | -0.157   | -0.096                                   |
| 4  | -0.212           | -0.212   | -0.215   | -0.215   | -0.154                                   |
| 5  | -0.271           | -0.272   | -0.277   | -0.277   | -0.207                                   |
| 6  | -0.330           | -0.331   | -0.339   | -0.339   | -0.262                                   |
| 7  | -0.389           | -0.391   | -0.401   | -0.404   | -0.321                                   |
| 8  | -0.447           | -0.453   | -0.462   | -0.469   | -0.380                                   |
| 9  | -0.494           | -0.510   | -0.511   | -0.523   | -0.438                                   |
| 10 | -0.525           | -0.554   | -0.542   | -0.571   | -0.491                                   |
| 11 | -0.556           | -0.576   | -0.575   | -0.594   | -0.519                                   |
| 12 | -0.565           | -0.579   | -0.584   | -0.598   | -0.525                                   |

Series 1 for the cystine-cysteine system at 25° is computed on the basis of the thermodynamic ionization constants, *i.e.* allowance made for activity coefficients, derived from the data of Sano (for cystine) of the authors' for cysteine; Series 1 at 37°, on the basis that the changes in the ionization constants of the two amino acids with temperature are such that the term in which they occur in the electrode equation has the same value at 25° and 37°, apart from the change in value of  $RT/2f$ . Series 2 is computed on the basis of Cannan and Knight's titration constants (29), *i.e.* no allowance made for activity coefficients, but corrected for temperature at 25° and 37° as suggested by Cohn. The values of the constants used, expressed as  $pK'$ , are:

| Series No. | Temperature | Cystine |         |         |         | Cysteine |         |         |
|------------|-------------|---------|---------|---------|---------|----------|---------|---------|
|            |             | $pK'_1$ | $pK'_2$ | $pK'_3$ | $pK'_4$ | $pK'_1$  | $pK'_2$ | $pK'_3$ |
| 1          | 25          | 1.04    | 2.05    | 8.00    | 10.25   | 1.71     | 8.33    | 10.78   |
| 1          | 37          | 1.04    | 2.05    | 8.00    | 10.25   | 1.71     | 8.33    | 10.78   |
| 2          | 25          | 1.0     | 1.72    | 7.65    | 9.19    | 1.96     | 8.35    | 10.28   |
| 2          | 37          | 1.0     | 1.72    | 7.23    | 8.77    | 1.96     | 7.93    | 10.28   |

$pK$  values from Cannan and Knight's data corrected for activity coefficients

|  |    |     |      |      |      |      |      |       |
|--|----|-----|------|------|------|------|------|-------|
|  | 30 | 1.0 | 1.64 | 7.57 | 9.42 | 1.76 | 8.26 | 11.00 |
|--|----|-----|------|------|------|------|------|-------|

as described above. The values so obtained for cysteine are in good agreement, after allowance is made for the temperature difference, with those found in our electrometric titrations. In the case of cystine there are large differences between the values obtained by Cannan and Knight and those deducible from the solubility data of Sano. These differences are quite large for  $pK_1$ ,  $pK_2$ , and  $pK_4$ . Accordingly we have computed the potentials at different hydrogen ion concentrations for the cystine-cysteine system, employing on the one hand the thermodynamic dissociation constants based upon our titration data for cysteine and the solubility data of Sano, and on the other the titration constants, *i.e.* not corrected for activity coefficients, of Cannan and Knight modified for the difference in temperature as suggested by Cohn (32). The figures in Table V show that the calculated values of the potentials in the range of hydrogen ion concentrations from pH 4 nearly to 8 are practically unaffected by differences in the values chosen for the ionization constants. In this range the term

$$\frac{RT}{nF} \ln \frac{(K_1^{SH})^2}{K_1^{SS} K_2^{SS}}$$

and the ionization term nearly cancel out, so that the equation as a close approximation becomes in this pH range

$$E_{obs.} = \bar{E} - 2.303 \frac{RT}{f} \text{ pH} \quad (10)$$

This numerical coincidence occurs in the range of hydrogen ion concentrations where both amino acids are predominantly in the zwitter ionic form.

At 37°  $\bar{E}$  is from Table I

$$\frac{-158,710 - 2(-80,110)}{2 \times 23,074} = +0.032 \text{ volt}$$

The large differences in the dissociation constants of cystine calculated from Sano's solubility data at 25° and the titration data of Cannan and Knight at 30° forbid their employment for the computation of the dissociation constants of cystine at 37°. So employed they lead to the extremely improbable values for the

heats of neutralization, computed by means of the equation  $\Delta pK = -\frac{\Delta H}{R \times 2.303} \left( \frac{T_2 - T_1}{T_1 T_2} \right)$ , of the second and fourth ionizable groups of 34,000 and 69,000 calories respectively. Accordingly the potentials at 37° for the cystine-cysteine system given in Table V are calculated on the basis that the changes in the ionization constants of the two amino acids with temperature are such that the term in which they occur in the electrode equation has the same value at 25° and 37°, apart from the change in value of  $RT/2f$ . The other series of values at 37° is computed on the basis of Cannan and Knight's titration constants corrected for temperature. The values of the constants used are given in the foot-note to Table VI. As at 25°, the values of the potentials from pH 4 to 8 are independent of these constants.

*Oxidation-Reduction Potentials of Dithiodilactic Acid  $\rightleftharpoons$  Thiolactic Acid System*—The electrode equation for the reaction  $\beta, \beta'$ -dithiodilactic acid +  $2H^+ + 2(e) \rightleftharpoons \beta$ -thiolactic acid is

$$E_{\text{obs}} = \bar{E} - \frac{RT}{2f} \ln \frac{(R-SH)^2}{(R-S-S-R)} - \frac{RT}{2f} \ln \frac{(H^+)^2 + K_1^{SS}(H^+) + K_1^{SS}K_2^{SS}}{((H^+)^2 + K_1^{SH}(H^+) + K_1^{SH}K_2^{SH})^2} \quad (11)$$

The significance of the symbols is similar to those in Equation 9.

$\bar{E}$  at 25° from Table I is

$$\frac{-165,900 - 2(-84,810)}{2 \times 23,074} = +0.081 \text{ volt}$$

By inserting this value of  $\bar{E}$  and the ionization constants of Table V into Equation 12 we have computed the  $E'_0$  values given in Table V.

The data in Table V show that the amino group strengthens considerably the reducing potential of the adjacent sulfhydryl group. Increasing the temperature from 25° to 37° slightly increases the reducing potential.

*Direct Experimental Measurements of Cystine  $\rightleftharpoons$  Cysteine Oxidation-Reduction Potential*—In Table VI are collected the potentials observed experimentally for the cystine-cysteine system from three different laboratories. In every case it was concluded that the system under observation was cystine +  $2H^+ + 2(e) \rightleftharpoons 2$  cys-

teine. These values are all markedly different from that calculated from the thermal data.

It has been shown (17) in the case of three enzymatic reactions that, when the experimental reaction under observation is the same as that postulated, the observed potentials are in close agreement with those based on thermal data in which the intervention of the enzyme is ignored. In the cystine-cysteine system there is the more reason for expecting close agreement between the observed potentials and those calculated from thermal data because the complication of the requirement of an enzyme for the

TABLE VI

$E''_0$  (in Volt) at 25° and pH 7.0 for  $L$ -Cystine +  $2H^+ + 2(e) \rightleftharpoons L$ -Cysteine

| Method   | $E''_0$ | Investigator                          |
|--|---------|---------------------------------------|
| Potentiometric oxidation of cysteine with                              |         |                                       |
| $KIO_3$ .....  | +0.06   | Williams and Drissen (33)             |
| $K_2Cr_2O_7$ .....   | +0.13   |                                       |
| $I_2$ .....  | +0.13   |                                       |
| Potentiometric; electrolytic formation of cysteine....                 | -0.329  | Ghosh, Raychaudhuri, and Ganguli (13) |
| Colorimetric; oxidation of cysteine, reduction of cystine by dyes..... | -0.222  | Fruton and Clarke (14)                |
| From thermal data.....   | -0.390  | Authors                               |

attainment of equilibrium in the experimental reaction was absent in all three of the above cases.

We have estimated that the extreme possible error in the thermal data for the free energy change in this reaction is 2000 calories. The probable error is much less. In terms of potential this extreme possible error is 43 millivolts. At pH 7.0 the potential calculated from the thermal data is practically independent of the dissociation constants assigned to the amino acids or of their activity coefficients. At hydrogen ion concentrations where these significantly affect the value of the calculated potential all other assumptions than those we have preferred (for independent reasons) augment the discrepancy between the calculated and observed potentials.

It is certain that the calculated potential corresponds to the reaction which has been formulated only in the case of the thermal value. The reason for the large discrepancies in Table VI must therefore be sought in the experimental conditions in which the different values were obtained. In two of the three studies under consideration here the data given show conclusively that the potentials observed were significantly dependent on other reactions which were not taken into account.

In the experiments of Williams and Drissen (33) the shape of the titration curves, and the variation of the  $E'_0$  values with the nature of the oxidizing agent indicate that what was actually measured, for example in the case of the iodine titration of cysteine, was the reduction potential of the system  $I_2 \rightleftharpoons 2I^-$ . This conclusion is corroborated by the behavior of cysteine toward dyes (14, 34). 0.01 *N* cysteine for example at 30° and pH 7.0 completely reduces indigomonosulfonate whose  $E'_0$  is -0.16 volt. This would be impossible if the values of Williams and Drissen were even approximately correct.

There can be little doubt that the potentials observed by Ghosh, Raychaudhuri, and Ganguli (13) refer to a system in which cystine and cysteine are participants. These authors varied the concentrations of cystine and cysteine by electrolytic reduction of the cystine at the same mercury electrodes which were used to measure the potentials. The electrolysis also achieves, according to Ghosh and Ganguli, the necessary removal of the film of oxygen on the electrode. The potentials which they observed have been confirmed quantitatively by Green (34). He also confirmed their finding that the potential is 60 millivolts more negative after electrolysis than before.

The experiments of Ghosh and Ganguli support their view that the failure of previous workers to obtain thermodynamically reversible electrode potentials with the cystine-cysteine system is to be ascribed to a film of oxygen so firmly bound on the mercury electrodes used that it can be removed only by electrolysis. But their experimental data indicate that complexes of mercury and cystine probably existed in their solutions. In Table VII are given the concentrations of cystine which they reported, and the solubility of cystine at the same hydrogen ion concentrations and temperatures calculated from dissociation and titration constants

and also interpolated from the directly observed solubilities of Sano. Ghosh *et al.* gave no details regarding the buffer solutions used and hence no allowance can be made for activity coefficients. In the pH range in which they worked, this correction is probably small, since very little of the cystine was in the divalent form, and their buffers were dilute (a large change in pH (0.6) occurred when the acid cystine solution was mixed with the buffer). The solubility of cystine in the solutions used by Ghosh *et al.* was probably therefore between those calculated from ionization

TABLE VII

*Comparison of Concentrations of Cystine Found by Ghosh et al., and Solubility of Cystine at Different Hydrogen Ion Concentrations and Temperatures*

| Temperature | pH   | Found by<br>Ghosh <i>et al.</i> | Interpolated<br>from Sano's<br>observed<br>solubilities | Calculated from  |  |
|-------------|------|---------------------------------|---|--|--|
|             |      |                                 |   | Ionisation<br>constants,<br>without<br>introduction<br>of activity<br>coefficients | Titration<br>constants of<br>Cannan and<br>Knight,<br>corrected for<br>temperature |
| °C.         |      | <i>mole per l.</i>              | <i>mole per l.</i>                                      | <i>mole per l.</i>   | <i>mole per l.</i>   |
| 25          | 8.60 | 0.0024                          | 0.0025  | 0.0023   | 0.0056   |
|             | 8.50 | 0.0024                          | 0.0021  | 0.0019   | 0.0043   |
|             | 8.50 | 0.0018                          | 0.0021  | 0.0019   | 0.0043   |
|             | 8.40 | 0.0024                          | 0.0018  | 0.0016   | 0.0034   |
|             | 8.40 | 0.0015                          | 0.0018  | 0.0016   | 0.0034   |
|             | 8.05 | 0.0018                          | 0.00076   | 0.00097  | 0.0017   |
|             | 8.05 | 0.00087                         | 0.00076   | 0.00097  | 0.0017   |
|             | 7.5  | 0.0034                          | 0.00069   | 0.00060  | 0.00079  |
|             | 7.5  | 0.0028                          | 0.00069   | 0.00060  | 0.00079  |
|             | 7.0  | 0.00171                         |   | 0.00043  | 0.00044  |
| 20          |      |                                 |   |  |  |

constants alone (*i.e.* without taking activity coefficients into account) and the values obtained by Sano in various buffer solutions. Table VII shows that the concentrations of cystine found by Ghosh *et al.* in some of their solutions exceeded the calculated and experimentally observed solubilities. They also exceed from pH 7.0 to 8.05 the solubilities computed from the titration constants of Cannan and Knight, which were obtained in solutions whose ionic strengths varied from 0.02 to 0.1. At pH 7.0 the discrepancy between the concentration of cystine found by Ghosh *et al.* and the expected solubility is greatest. At this pH uncer-

tainties regarding dissociation constants and activity coefficients affect the value of the calculated solubility only to a slight extent. If the pH values of the solutions of Ghosh *et al.* were those reported, it follows that a large fraction of the cystine (supersaturation being excluded) was not free in solution. The obvious suggestion is that it existed in the form of a mercury-cystine complex.

An alternative hypothesis to complex formation, to account for the discrepancy between the solubility data and the concentration of cystine found by Ghosh *et al.*, is that in the course of the electrolysis, not only was cysteine formed, but the solutions also became much more alkaline. This possibility is excluded by the concordance of the  $E_0$  values obtained by Ghosh, Raychaudhuri, and Ganguli, by Green, and by Ghosh and Ganguli, calculated on the basis of initial pH (before electrolysis) in buffer solutions ranging from pH 7.0 to 9.2, and secondly by the observation of Green, that an electrolyzed solution gave the usual more positive potential against fresh mercury.

In spite of the fact, therefore, that Ghosh *et al.* observed no precipitate when they passed  $H_2S$  through their solutions, their data indicate that a mercury complex of cystine, and probably also therefore of cysteine, existed in their solutions. As a result their standard electrode potential,  $E_0$ , is composed not only of the molal electrode potential of the cystine-cysteine reaction, and the log of the ratio of their ionization constants, but also of a term for the ratio of the equilibrium constants of the two amino acids with mercury. It is improbable that the equilibria between cystine and cysteine and mercury are such that they exactly cancel each other.

We must call attention here to the combustion values for cystine and cysteine reported by Becker and Roth (35). These are lower than those given in Table I obtained in this laboratory. They yield standard free energy values,  $\Delta F^0$ , of  $-82,480$  and  $-166,630$  for cysteine and cystine respectively, which lead to a value of  $+0.096$  volt for  $\bar{E}$  instead of  $+0.025$  volt calculated from the thermal data in Table I. The higher figure for  $\bar{E}$  gives  $E''_0$  values in reasonably close agreement with those given of Ghosh *et al.* Thus at pH 7.0,  $E''_0$  from our thermal data is  $-0.390$  volt, according to Ghosh *et al.* it is  $-0.329$  volt, and from the combustion data of Becker and Roth it is  $-0.319$  volt.



Nevertheless this agreement in the last two values is only fortuitous. In a private communication Professor Roth agreed that their combustion values are less reliable than ours. Only a small amount of material was supplied them, and they burned it without either testing its purity or purifying it further. That it contained considerable impurity is indicated by the lower percentages of sulfur which they found, by their lower values for the heats of combustion, and by the large variations in the individual determinations.

Fruton and Clarke (14) measured the potential of the cystine-cysteine system colorimetrically. They measured the equilibrium degree of reduction of dyes with known oxidation-reduction potentials by different concentrations of cysteine at different hydrogen ion concentrations. The following evidence attested to the thermodynamic reversibility and the accuracy of the observed potentials. The same  $E'_0$  values (corresponding to  $E''_0$  in Tables VI and VII) were obtained with different dyes and with different concentrations of cysteine. The dyes in their reduced form were reoxidized by cystine, and the final degree of reoxidation attained was in accord with the  $E'_0$  values given by the reduction experiments with cysteine.

From these observations the  $E''_0$  value of the cystine-cysteine system at pH 7.0 and 25° is  $-0.22$  volt, which is 100 millivolts more positive than the value given by Ghosh *et al.* and 170 millivolts more positive than our thermal value.

These discrepancies are far beyond the possible experimental errors. We accordingly undertook a repetition of the experiments of Fruton and Clarke, and have confirmed them in principle, though we have been unable to confirm them in detail. We have found that an equilibrium is attained *in vacuo*, of which at least some, if not all, of the components are cysteine, cystine, and the oxidized and reduced forms of the dye. The  $E''_0$  values we obtained were approximately 50 millivolts more negative than those reported by Fruton and Clarke.

We shall describe only the general features of our experimental procedure which was not essentially different in the reduction experiments from that of Fruton and Clarke. The cysteine was measured into the overhang of a Thunberg tube and the other solutions—dye, buffer, and cystine—into the lower compartment.

The vessel was evacuated and then the contents of the upper and lower compartments were mixed. The vessels containing different mixtures were then kept in a vacuum desiccator which was continuously evacuated by an oil pump. The dyes and amino acids used were recrystallized specimens. The dyes finally chosen, indigodisulfonate and brilliant alizarin blue, were used in concentrations of 0.00005 M and 0.0001 M. The final concentration of cysteine was varied from 0.0001 to 0.001 M, and of cystine up to 0.002 M. The experiments were carried out at room temperature and in buffer solutions at pH 7.5 (phosphate), 8.0, and 8.5 (borate). After equilibrium was attained, in 2 to 3 days, the vacuum in the desiccator was broken by admitting purified hydrogen or nitrogen, and the colors in the Thunberg tubes compared with previously prepared dilutions of the dye. The desiccator technique with continuous evacuation was used because we were unable otherwise to maintain a sufficiently high vacuum for the length of time required for the attainment of equilibrium. With these low concentrations of reactants an air leak giving a pressure of 1 mm. of Hg radically distorted the results.

We observed that in solutions containing the same concentration of cysteine and different concentrations of added cystine that the degree of reduction finally attained was always less the higher the initial concentration of cystine. This was the case with brilliant alizarin blue and with indigodisulfonate. On the assumption that the reaction here was simply 2 cysteine + oxidized dye  $\rightleftharpoons$  cystine + reduced dye the calculated  $E''_0$  value at pH 8.0 was between  $-0.29$  and  $-0.30$ . The corresponding value of Fruton and Clarke at 25° is approximately  $-0.27$  volt. At pH 8.5, with brilliant alizarin blue, the  $E''_0$  value we have found was between  $-0.32$  and  $-0.34$ ; that of Fruton and Clarke is  $-0.29$  volt.

In most of the experiments the effect of the added cystine on the equilibrium degree of reduction was less than the theoretical amount calculated on the basis of the reaction 2 cysteine + reduced dye  $\rightleftharpoons$  cystine + oxidized dye. We do not at present attach more than semiquantitative significance to these observations and the above  $E''_0$  values. The range of concentrations of cysteine, which gave degrees of reduction which were neither too low nor too high to be measured, was small, between 0.0004 M and 0.0007 M, with 0.0001 M indigodisulfonate; the maximum possible concentration

of cystine at pH 8.5 is only approximately 0.002  $m$ ; and the method for measuring the color is crude.

In every instance, with and without added cystine, and with indigodisulfonate, brilliant alizarin blue, and cresyl violet, we observed a greater degree of reduction than the  $E''_0$  values of Fruton and Clarke call for. In further disagreement with their observations we have been unable to observe any reoxidation of reduced dyes by cystine with concentrations of reactants and hydrogen ion concentration identical with theirs.

Nevertheless, in confirmation of Fruton and Clarke's observations, the dye-reducing potency of cysteine, which we have observed, fell far short of that called for by the  $E''_0$  values of Ghosh *et al.*, let alone the thermal values. The present situation of this problem may be summarized therefore as follows: Two experimental systems have been found, those of Ghosh *et al.* and of Fruton and Clarke, in which cysteine and cystine participate in an oxidation-reduction equilibrium. The potentials obtained in these two systems differ by 100 millivolts, much too large a quantity to be ascribed to experimental error. Both these directly obtained experimental values are more positive than the potential calculated from thermal data. The potential of Ghosh *et al.* is nearer to the thermal value, but even it differs by 70 millivolts. This difference is within the possible error of the thermal data. Yet the experimental data presented by Ghosh *et al.* show conclusively that important reactions were not taken into account in their calculation of the potential. In view of the even greater difference, 170 millivolts, between the colorimetric and thermal values, it is highly probable that in these colorimetric measurements also there are important side reactions. The only possible doubt of the thermal value is regarding the accuracy of the actual experimental measurements. It is improbable that any corrections here would bring it into accord with one of the experimental values. While, as stated above, the final reference value for the stated reaction is the thermal value, its usefulness is limited. In any set of experimental conditions the experimental oxidation-reduction potential may be more pertinent. But the difference between it and the thermal value is an indication that the reaction is not simply the reduction of cystine to cysteine or *vice versa*.

*Catalytic Action of Selenium Dioxide on Oxidation of Cysteine—*

In the course of our colorimetric experiments we observed that small amounts of selenium dioxide catalyze the reduction of indigodisulfonate by cysteine. Thus with 0.00005 M dye, 0.001 M cysteine, and 0.00001 M  $\text{SeO}_2$ , the dye is completely reduced in 1 hour at room temperature, whereas without the selenium dioxide nearly a week is required. Salts of iron, copper, cobalt, nickel, or magnesium do not possess this catalytic property. With high concentrations of cysteine (0.1 M) and  $\text{SeO}_2$  (0.01 M) a white crystalline complex precipitates out of solution. Dissolved in strong HCl, red selenium and cystine precipitate out. The same degree of reduction of dyes was attained with selenium dioxide as without, both with and without added cystine. The only dyes we could use with this catalyst in the range of hydrogen ion concentrations from pH 7.0 to 8.5 were indigodisulfonate and phenosafranin. Brilliant alizarin blue, gallophenine, and cresyl violet appeared to be destroyed by it.

#### SUMMARY

1. Data are presented from which the oxidation-reduction potentials of the systems, cystine  $\rightleftharpoons$  cysteine, and dithiodilactic acid  $\rightleftharpoons$  thiolactic acid, have been calculated.

2. These values and other evidence show that the potentials previously obtained for the cystine  $\rightleftharpoons$  cysteine system by direct measurement are not referable exclusively to this system.

3. Some experiments are described in which addition of cystine has been observed to affect the degree of reduction of dyes by cysteine.

4. Selenium dioxide is a catalyst for the reduction of indigodisulfonate (and probably other dyes not attacked by the selenium dioxide) by cysteine. A cysteine-selenium dioxide complex has been isolated.

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# THE ACTION OF MERCURIC SULFATE AND CHLORIDE ON CYSTEINE, CYSTINE, CYSTEINE SULFINIC ACID ( $R-SO_2H$ ), AND CYSTEIC ACID WITH REFERENCE TO THE DISMUTATION OF CYSTINE\*

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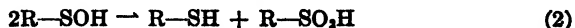
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Preceding studies (1) show that one of the products of the dismutation of cystine disulfoxide ( $R-(SO)_2-R$ ) is the sulfinic acid ( $R-SO_2H$ ), a compound which possesses greater stability in solution than any other intermediate oxidation product of cystine obtained thus far. It was interesting, therefore, to determine whether the sulfinic acid results from the dismutation of cystine in the presence of heavy metal salts, especially since published data can be so interpreted.

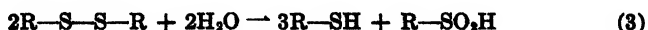
It becomes increasingly evident that many reactions of cystine can be best explained by first assuming that hydrolysis occurs



In the presence of heavy metal salts, cysteine is eliminated by precipitation, thus leaving the sulfenic acid free to dismute



If, as postulated, the dismutation stops with the sulfinic acid—and as yet there is no evidence of dismutation of the sulfinic acid<sup>1</sup>—then the dismutation of cystine can be expressed by the following stoichiometrical equation




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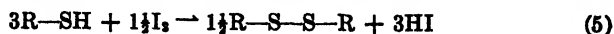
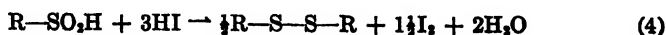
\* Aided by a grant from the Leffmann Fund of the Wagner Free Institute, Philadelphia.

<sup>1</sup> There is some evidence that cysteic acid is produced by the dismutation of cystine disulfoxide in acid solution (1).

with 75 per cent of the cystine being converted to cysteine and 25 to the sulfinic acid. Since the sulfinic acid is an intermediate oxidation product which can, under suitable conditions, be either reduced to cystine or oxidized to cysteic acid, there are various means which can be employed to determine whether or not the dismutation proceeds according to Reaction 3.

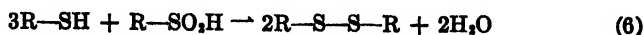
The dismutation was effected in the present studies by mixing sulfuric acid solutions of cystine and mercuric sulfate. The white precipitate which formed was filtered off and resuspended in water, the mercury removed as  $\text{HgS}$ , and the excess  $\text{H}_2\text{S}$  expelled from the solution by  $\text{CO}_2$  or  $\text{N}_2$ . The colorimetric determination of cysteine in the solution, by Shinohara's modification of the phospho-18-tungstic acid procedure (2), a test which is not affected by the sulfinic acid, showed that 75 per cent of the original cystine had been converted to cysteine.

Direct confirmation of this result by iodometric oxidation of cysteine to cystine in  $\text{KI-HCl}$  solution (3) is not possible, since the sulfinic acid is here reduced to cystine with the liberation of  $\text{I}_2$ . Indeed, a solution containing cysteine and sulfinic acid in a molar ratio of 3:1, such as would be produced by Reaction 3, will neither consume nor liberate  $\text{I}_2$ , even in the presence of excess  $\text{I}_2$ , when it is made 2 M in  $\text{KI-HCl}$ , as shown by Reactions 4 and 5.



This fact which was confirmed with the above solution of the dismutation products of cystine may be accepted as indicating the presence of an intermediate oxidation product.

The summation of Reactions 4 and 5 results in Reaction 6



which is seen to be the reverse of the original dismutation Reaction 3, and which is free to proceed after the heavy metal has been removed. Accordingly, owing to the formation of cystine, the optical rotation of such solutions becomes increasingly negative (1) and, as might be expected from Reactions 4 and 5, the rate of Reaction 6 is accelerated by iodide ions.

After oxidation of cysteine to cystine by aeration, the sulfinic

acid present was determined by the amount of  $I_2$  liberated in 2 M KI-HCl solution (since cystine in contrast to cysteine does not consume  $I_2$  under these conditions). The sulfinic acid can apparently withstand aeration and also evaporation on a water bath, since in some cases the theoretical amount of  $I_2$  was liberated. The acidity of the solution also suggests the formation of the sulfinic acid. The attempts to isolate both a barium salt and the free acid resulted in impure products which were not positively identified.

The estimation of total cysteine and sulfinic acid (or after air oxidation, total cystine and sulfinic acid) by means of the  $I_2$  required for oxidation of the two compounds to cysteic acid (4) showed approximately 94 per cent of the theoretical. The optical rotation of *cystine* solutions obtained by adding KI to the original dismutation mixture (Reactions 4 and 5) and subsequently removing Hg by extraction with ether (as  $HgI_2 \cdot HI$ ) indicated recovery of only 90 per cent of the original cystine; iodometrically, 92 to 98 per cent recovery was indicated.

Cysteine, cysteic acid, and the sulfinic acid were also precipitated individually by mercuric sulfate from 2 N  $H_2SO_4$  solution. The behavior of the above compounds as well as cystine with mercuric chloride was also investigated.

#### EXPERIMENTAL

*Cysteine and Mercuric Sulfate*—The addition of a solution containing 8.05 mm of  $HgSO_4$  in 11 cc. of 1.9 N  $H_2SO_4$  to a solution of 4 mm of cysteine (5) in 6 cc. of 1.9 N  $H_2SO_4$  resulted in the formation of a white precipitate which after standing 2 hours was filtered off by suction, washed with water, alcohol, and ether, and dried *in vacuo* over  $H_2SO_4$  at room temperature; yield 2.276 gm. The completeness with which cysteine is precipitated was shown by the fact that only 0.003 mm remained in the filtrate, which also contained 0.973 milli-atom of  $Hg^{++}$ . The precipitate was analyzed as follows: 0.500 gm. was suspended in  $H_2O$  and  $Hg^{++}$  removed and weighed as  $HgS$  (total of 7.18 milli-atoms of  $Hg^{++}$ ); after  $H_2S$  was expelled from the filtrate by a current of  $N_2$ , aliquots were taken and cysteine determined both colorimetrically (4.05 mm of R—SH) and by  $I_2$  oxidation in 1 M KI-HCl (3.96 mm of R—SH). The  $H_2SO_4$  in the remainder of the filtrate was titrated



with NaOH with methyl red as indicator (3.76 mm of  $\text{H}_2\text{SO}_4$ ) and was subsequently precipitated and weighed as  $\text{BaSO}_4$  (3.84 mm of  $\text{BaSO}_4$ ). The analysis indicated the following molecular proportions  $1\text{R}-\text{S}^- : 1.8\text{Hg}^{++} : 0.96\text{SO}_4^-$ , corresponding to a total weight equal to 101 per cent of that of the precipitate. No evidence of the formation of  $\text{Hg}^+$  was obtained; i.e., no blackening with KI or  $\text{NH}_4\text{OH}$  and no  $\text{HgCl}$  formation when dissolved in HCl.

The optical rotation,  $\alpha_{\text{Hg}}^{25}$ , of a solution of 0.5 gm. of the precipitate (approximately 0.105 gm. of cysteine) in 10 cc. of  $\text{M}$  HCl was  $+0.79^\circ$  per dm. On standing, this solution deposited dense tufts of crystals (cf.  $\text{HgCl}_2$  below). After the addition of 0.5 gm. of the original precipitate to 5 cc. of  $2\text{ M}$  HCl, followed by the addition of 6.32 mm of KI (4 times the amount of  $\text{Hg}^{++}$  present) the mixture was diluted to 10 cc. and the mercury removed by extraction with ether (as  $\text{HgI}_2 \cdot \text{HI}$ ). The resulting aqueous solution now possessed a rotation,  $\alpha_{\text{Hg}}^{25}$ , of  $+0.088^\circ$  per dm. in agreement with that of cysteine.

*Cystic Acid and Mercuric Sulfate*—Cystic acid was prepared by the oxidation of cystine with  $\text{Br}_2$ ; the equivalent weight of this material determined by titration with NaOH, with methyl red as indicator, amounted to 174 gm. instead of the theoretical 169 gm. Mixture of a solution of 4.06 mm of  $\text{R}-\text{SO}_3\text{H}$  (0.708 gm.) in 5 cc. of  $1.9\text{ N}$   $\text{H}_2\text{SO}_4$  with a solution of 8 mm of  $\text{HgSO}_4$  in 12 cc. of  $1.9\text{ N}$   $\text{H}_2\text{SO}_4$  caused a heavy white precipitate to form. After standing  $1\frac{1}{2}$  hours, the mixture was filtered by suction, washed once with  $2\text{ N}$   $\text{H}_2\text{SO}_4$ , 50 per cent alcohol and water, alcohol, and finally ether, and dried *in vacuo* over  $\text{H}_2\text{SO}_4$  at room temperature. A small additional amount (0.122 gm.) precipitated from the filtrate on standing; total weight of the precipitate, 1.312 gm. This precipitate is semicrystalline, appearing as small blades, and dissolves quite easily in chloride solutions, viz.  $\text{NH}_4\text{Cl}$ ,  $\text{BaCl}_2$ , and HCl. Analysis, similar to that of the cysteine precipitate, showed 3.22 milli-atoms of  $\text{Hg}^{++}$ , 4.11 milli-equivalents of acid, and 0.17 mm of  $\text{SO}_4^-$  to be present. Deduction of the  $\text{SO}_4^-$  (which probably represents contamination) from the total acid leaves 3.77 mm of  $\text{R}-\text{SO}_3\text{H}$  as occurring in the precipitate or about 94 per cent the amount used. The molecular ratio of  $1\text{Hg}^{++} : 1.17\text{R}-\text{SO}_3^- : 0.053\text{SO}_4^-$  which was obtained indicates 100.2 per cent of the

weight of the precipitate. The rotation,  $\alpha_{\text{H}_2\text{O}}^{25}$ , of 0.19 gm. of the precipitate (containing 0.095 gm. of cysteic acid according to the analytical evidence) dissolved in 10 cc. of  $\text{M HCl}$  was  $+0.118^\circ$  per dm., in agreement with the figure  $+0.116^\circ$  obtained with a solution of 0.1 gm. of cysteic acid in 10 cc. of  $\text{M HCl}$ .

*Sulfinic Acid and Mercuric Sulfate; Some Observations on the Sulfinic Acid*—The procedure previously described for the preparation of the sulfinic acid by ammoniacal decomposition of cystine disulfoxide (1) apparently gives rise to several hydrates. When the solution containing the sulfinic acid was evaporated to small volume, crystals were deposited in the form of small, crossed, elongated hexagons. These crystals, after drying on a porous plate or between filter papers in a desiccator over  $\text{CaCl}_2$ , yielded the following equivalent weights (*cf.* (1)): 188.5 by titration with alkali; 180.7 by reduction in 2  $\text{M KI-HCl}$ ; 183.1 by  $\text{I}_2$  oxidation; m.p.  $146^\circ$ . Although the substance becomes discolored on drying *in vacuo* at  $100^\circ$ , there was no loss in weight. The above figures suggest the dihydrate,  $\text{R-SO}_2\text{H} \cdot 2\text{H}_2\text{O}$ ; molecular weight 189.

In the previously reported preparation (1) crystals of the non-hydrated acid ( $\text{R-SO}_2\text{H}$ ) were deposited when a hot 50 per cent alcohol solution was cooled. However, this treatment usually produces two liquid layers, of which the lower can be solidified by stirring to loose, slightly yellow crystals. This material after being filtered off, washed with alcohol and ether, and dried *in vacuo* over  $\text{H}_2\text{SO}_4$  at room temperature yielded the following data: molecular weight 171.5 by titration with alkali, 161.3 by  $\text{KI-HCl}$  reduction, 144.2 by  $\text{I}_2$  oxidation; m.p.  $143^\circ$  (with decomposition). The specific rotation,  $[\alpha]_{\text{H}_2\text{O}}^{25}$ , of a solution containing 1 gm. in 100 cc. of  $\text{N HCl}$  was  $+29.3^\circ$ ; if the substance is assumed to be the monohydrate  $\text{R-SO}_2\text{H} \cdot \text{H}_2\text{O}$ , the value is  $+32.7^\circ$ , in fair agreement with the value  $+33.4^\circ$  previously obtained for the sulfinic acid  $\text{R-SO}_2\text{H}(1)$ . Although the analytical data are not very satisfactory, since the acid titration indicates a monohydrate  $\text{R-SO}_2\text{H} \cdot \text{H}_2\text{O}$  (mol. wt. 171) and the other data  $\text{R-SO}_2\text{H}$  (mol. wt. 153), nevertheless the material was used as the best available, especially since reprecipitation by alcohol did not improve the results. Mention should be made of the fact that the sulfinic acid consumes  $\text{I}_2$  relatively rapidly, which may be a consideration in iodometric determinations, when the sulfinic acid

might be present. Thus, although the acid cannot be titrated directly with  $I_2$ , in the presence of a 4-fold excess of  $I_2$  back titration of the excess with  $Na_2S_2O_3$  yielded the following molecular weights: 152.5 after 20 minutes (*i.e.* theoretical for  $R-SO_2H$ ), 146 after 1 hour, and 144.2 after 48 hours. The difference between the last two figures may be attributed to the 0.5 per cent cystine which was present and which is more slowly oxidized to cysteic acid.

The addition of 0.855 gm. of the above sulfinic acid (5 mm, with a molecular weight of 171) dissolved in 8 cc. of 1.9 N  $H_2SO_4$  to a solution of 3.0 gm. of  $HgSO_4$  (10 mm) in 12 cc. of 1.9 N  $H_2SO_4$  caused the immediate formation of a thick, white paste. After standing overnight, the mixture was filtered by suction, washed once with 1.9 N  $H_2SO_4$ , with 50 per cent alcohol, with alcohol, and with ether, and dried *in vacuo*; yield 2.36 gm. The addition of  $NH_4Cl$  to the filtrate precipitated a white flocculent precipitate (0.0928 gm.) which, when titrated with  $I_2$  containing KI, consumed only 0.292 milli-equivalent of  $I_2$  instead of the theoretical 0.39 milli-equivalent required by the precipitate if it were  $HgCl$ . After the  $Hg^{++}$  remaining in the filtrate (3.30 mm) was removed with  $H_2S$ , it was necessary to bubble  $N_2$  through the solution for 10 hours before a negative test with lead acetate paper could be obtained. 0.365 mm of sulfinic acid remained in the filtrate, indicating precipitation of about 93 per cent.

The precipitate, which is hygroscopic, contained 6.29 milli-atoms of  $Hg^{++}$ , 4.69 mm of  $R-SO_2H$ , 11.93 milli-equivalents of acid titrated by  $NaOH$ , and 3.75 mm of sulfate; deduction of the sulfate from the total acid indicates 4.43 mm of  $R-SO_2H$  to be present. The analysis indicates the following molecular proportions,  $1R-SO_2^-:1.34 Hg^{++}:0.80 SO_4^{--}$ . The precipitate dissolves in solutions of  $NH_4Cl$ ,  $HCl$ , and  $BaCl_2$  (precipitation of  $BaSO_4$  occurs in the latter case). 0.4015 gm. dissolved in 10 cc. of M  $HCl$  and filtered from 0.0038 gm. of a white precipitate possessed an optical rotation,  $\alpha_{H_2O}^{25}$ , amounting to  $+0.39^\circ$  per dm. When  $+0.29^\circ$  is used as the rotation per dm. of 0.1 gm., the above figure indicates 0.781 gm. of sulfinic acid to be present in the precipitate or 91.5 per cent of that used.

The extraction of  $Hg$  with ether after addition of KI did not yield a solution whose rotation could be taken, because of con-

tinued liberation of  $I_2$ . No  $Hg^+$  was indicated upon addition of KI.

*Cystine and  $HgSO_4$ ; Determination of Cystine Colorimetrically and Iodometrically, and Estimation of Cystine Plus Intermediates*—0.300 gm. (2.5 milli-equivalents) of cystine dissolved in 5 cc. of 1.9 N  $H_2SO_4$  was added to 1.50 gm. of  $HgSO_4$  (5.0 mm) in 8.2 cc. of 1.9 N  $H_2SO_4$  contained in a 50 cc. volumetric flask. The mixture containing the white precipitate was diluted to the mark with water,  $H_2S$  passed in,  $HgS$  removed by filtration with suction (no washing), and the excess  $H_2S$  removed from the filtrate by a current of  $N_2$ . The amount of cystine present, determined colorimetrically with Shinohara's modification (2) of the phospho-18-tungstic acid test, was 1.950 mm or 78 per cent of the cystine used. However, when 5 cc. of 0.1 N  $I_2$  was added to a 10 cc. aliquot containing 5 cc. of 10 N HCl and 10 cc. of 5 N KI (total volume 50 cc.), there was no  $I_2$  consumed after 2 hours (compared with a corresponding blank).<sup>2</sup> In view of the cystine present this can only mean that the amount of  $I_2$  necessary for oxidation of cystine to cystine was liberated by reduction of some intermediate oxidation product to cystine.

The total  $I_2$  consumed in oxidation to cysteic acid by an excess of  $I_2$  in weakly acid solution (3) amounted to 5.90 mm or 94.5 per cent of the theoretical value for the calculated amounts of  $R-SH$  and  $R-SO_2H$ , i.e. 6.25 mm.

*Recovery of Cystine Polarimetrically and Reaction of Cystine with Sulfinic Acid*—2.5 milli-equivalents of cystine were dissolved in 4 cc. of 2.2 N  $H_2SO_4$ , contained in a 25 cc. volumetric flask, and 5.0 mm of  $HgSO_4$  in 8 cc. of 2.2 N  $H_2SO_4$  were added. The mixture was shaken for 5 minutes, 4 cc. of 5 M KI (20 mm) were added, the mixture was diluted to 25 cc., and the mercury extracted with two 25 cc. portions of ether.<sup>3</sup> The rotation,  $\alpha_{H_2O}^{25}$ , of the

<sup>2</sup> Because of the time required for reduction of the sulfinic acid, it is necessary to wait 1 or 2 hours in this test before determining the  $I_2$  consumed or liberated (1); thus in the above instance 0.236 milli-equivalent of  $I_2$  was consumed immediately after mixing, indicating a total of 1.18 mm of cysteine.

<sup>3</sup> Titration of the ether extracts with NaOH to the turning point of methyl red indicated that an amount of acid equivalent to the mercury was extracted from the aqueous solution; viz., 4.6 milli-equivalents from the dismutation solution, 4.9 milli-equivalents from the cystine blank, and 5.0 milli-equivalents from the cystine solution ( $HgI_2 \cdot HI$ ).

clear solution immediately after extraction amounted to  $-2.24^\circ$  per dm., which changed to  $-2.56^\circ$  on standing overnight and remained constant 24 hours later. A blank of 2.5 milli-equivalents of cystine, 5 mm of  $\text{HgI}_2$ , and 10 mm of KI treated in the same way yielded  $-2.85^\circ$  per dm. The rotation therefore indicates 90 per cent recovery of cystine (90 and 92 per cent in other experiments).

The  $\text{I}_2$  consumed by cystine in aliquots of the two solutions on

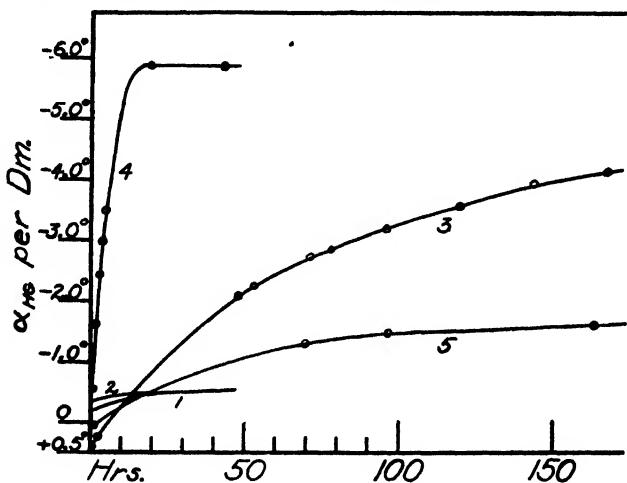


FIG. 1. Illustrating the reaction  $\text{R-SO}_3\text{H} + 3\text{R-SH} \rightarrow 2\text{R-S-S-R}$ . Solution 1, 0.15 M  $\text{R-SH}$ , 0.05 M  $\text{R-SO}_3\text{H}$ ; Solution 2, 0.15 M  $\text{R-SNa}$ , 0.05 M  $\text{R-SO}_3\text{Na}$ ; Solution 3, 0.15 M  $\text{R-SH}$ , 0.05 M  $\text{R-SO}_3\text{H}$ , 1 M  $\text{HCl}$ ; Solution 4, 0.15 M  $\text{R-SH}$ , 0.05 M  $\text{R-SO}_3\text{H}$ , 1 M  $\text{HCl}$ , 0.01 M  $\text{KI}$ ; Solution 5, filtrate from 0.5 gm. of dismutation precipitate in 10 cc. of M  $\text{HCl}$  after removal of  $\text{Hg}$  by  $\text{H}_2\text{S}$ .  $\alpha_{\text{H}_2\text{O}}^{20} = -5.95^\circ$  per dm. for 0.1 M  $\text{R-S-S-R}$  in M  $\text{HCl}$ . Cystine precipitated from Solutions 1 and 2 after 48 hours.

oxidation to cysteic acid by an excess of  $\text{I}_2$  indicated 0.0110 gm. of cystine per cc. of the dismutation solution and 0.0120 gm. of cystine per cc. of the blank or 91.8 and 100 per cent recovery, respectively; in another case 98 per cent recovery was indicated by the  $\text{I}_2$  oxidation.

Although these figures lack quantitative precision, nevertheless they are in such striking contrast to those obtained from similarly treated cysteine solutions that something other than iodide is clearly responsible for the formation of cystine. Thus 2.5 mm of

cysteine treated as above with 5 mm of  $\text{HgSO}_4$  and 20 mm of KI yielded after extraction with ether a solution with an optical rotation,  $\alpha_{\text{Hg}}^{24}$ , equal to  $+0.13^\circ$  per dm., which remained practically unchanged for 48 hours ( $+0.12^\circ$ ).

TABLE I

*Precipitation of Cystine by  $\text{HgSO}_4$* 

40 milli-equivalents of  $\text{R-S-S-R}$  in 60 cc. of 2.2 N  $\text{H}_2\text{SO}_4$  and 80 mm of  $\text{HgSO}_4$  in 90 cc. of 2.2 N  $\text{H}_2\text{SO}_4$  were mixed.

In Preparation 1,  $\text{HgSO}_4$  was added to  $\text{R-S-S-R}$  and the mixture allowed to stand for 3 days; in Preparation 2, the mixture was filtered 2 hours after mixing; in Preparation 3, cystine was added to  $\text{HgSO}_4$  and the mixture filtered after 2 days.

| Preparation No. ....                             | 1     | 2     | 3     | Theoretical |
|--|-------|-------|-------|-------------|
| Filtrate   |       |       |       |             |
| $\text{Hg}^+$ , milli-atoms.....                 | 3.75  | 3.72  | 3.97  |             |
| $\text{Hg}^{++}$ " .....                         | 10.2  | 10.7  | 6.9   |             |
| $\text{R-SH}$ , mm .....                         | 0.085 | 0.026 | 0.024 |             |
| $\text{R-SO}_2\text{H}$ " .....                  | 0.65  | 0.27  | 0.53  |             |
| Precipitate                                      |       |       |       |             |
| Weight, gm.....                                  | 22.0  | 21.8  | 22.7  |             |
| $\text{Hg}^+$ , milli-atoms. ....                | 8.7   | 11.8* | 18.2† |             |
| $\text{Hg}^{++}$ " .....                         | 55.5  | 53.4  | 51.6  |             |
| Total Hg " .....                                 | 78.2  | 79.6  | 80.7  | 80.0        |
| $\text{R-SH}$ , colorimetric, mm.....            | 29.9  | 29.7  | 27.4  | 30.0        |
| " $\text{I}_2 + \text{HI}$ " .....               | 13.0  | 5.7   | 0     | 0           |
| $\text{R-SO}_2\text{H}$ , calculated from        |       |       |       |             |
| $\text{R-SH} + \text{I}_2$ , mm.....             | 5.6   | 8.2   | 10.6  | 10.0        |
| $\text{R-SO}_2\text{H}$ (HI reduction), mm.....  | 7.0   | 9.8   | 9.5   | 10.0        |
| Total acid- $\text{H}_2\text{SO}_4$ , m.-eq..... |       | 6.7   | 9.5   | 10.0        |
| Sulfate ( $\text{H}_2\text{SO}_4$ ) " .....      |       | 69.2  | 79.5  |             |
| $\alpha_{\text{Hg}}^{24}$ per dm.‡, degrees..... | +0.65 | +0.59 | +0.65 |             |

\* 12.5 mm of  $\text{HgCl}_2$  by titration with  $\text{I}_2 + \text{KI}$ .

† 18.4 mm of  $\text{HgCl}_2$  by titration with  $\text{I}_2 + \text{KI}$ .

‡ 0.5 gm. of the precipitate dissolved in 10 cc. of M HCl.

This difference in behavior is attributed to the previously noted (1) reaction between cystine and the sulfinic acid (Reaction 6) which proceeds in the solution of the dismutation products of cystine after the mercury has been removed. This reaction is illustrated in Fig. 1 which shows the influence of acidity on the

reaction rate as well as the accelerating action of iodide (Reactions 4 and 5). The change in rotation of solutions from which the mercury was removed by  $\text{H}_2\text{S}$  was so slow that it was not utilized for quantitative purposes; one such solution from the precipitate Preparation 2 of Table I is shown in Fig. 1.

Table I contains additional data on the precipitation of cystine by  $\text{HgSO}_4$ .  $\text{Hg}^+$  was determined in the filtrate by adding  $\text{NH}_4\text{Cl}$  and weighing the precipitated  $\text{HgCl}$ ; the  $\text{Hg}^+$  in the precipitate from the dismutation was determined by dissolving 0.5 gm. in 10 cc. of  $\text{M HCl}$ , the precipitated  $\text{HgCl}$  was filtered off, dried, and weighed, and the weight confirmed by titration with  $\text{I}_2$  containing  $\text{KI}$ . The polarimetric data were obtained on this filtrate.  $\text{Hg}^{++}$  was precipitated as  $\text{HgS}$  after removal of  $\text{Hg}^+$ ; the total  $\text{Hg}$  recovered was nearly quantitative. After the excess  $\text{H}_2\text{S}$  had been expelled from the filtrate with  $\text{N}_2$ , the cysteine was determined colorimetrically as before. Titration of the cysteine in  $\text{M KI-HCl}$  solution with excess  $\text{I}_2$  yielded very low results; on the assumption that  $1\text{R-SO}_2\text{H}$  liberates  $\text{I}_2$  equivalent to  $3\text{R-SH}$ , and using the  $\text{R-SH}$  content obtained colorimetrically, one can calculate the amount of  $\text{R-SO}_2\text{H}$  present, as shown in Table I. Neutralization of the solution to the turning point of methyl red yielded the total acid content ( $\text{R-SO}_2\text{H} + \text{H}_2\text{SO}_4$ ). Aeration at pH 7 to 8 converted  $\text{R-SH}$  to  $\text{R-S-S-R}$ , after which the  $\text{R-SO}_2\text{H}$  was determined by reduction with  $2\text{M KI-HCl}$ . Deduction of sulfate, determined as  $\text{BaSO}_4$ , from the total acid also gives information as to the amount of sulfinic acid that formed. Removal of  $\text{Hg}$  by extraction with ether (as  $\text{HgI}_2\cdot\text{HI}$ ) resulted in solutions which possessed rotations similar to those of the preceding preliminary experiments.

*Isolation of Sulfinic Acid*—This was complicated not only by the difficulty of removing  $\text{Hg}$ , sulfate, and cysteine from the precipitate of cystine dismutation products without loss of sulfinic acid but also by the solubility and difficulty of crystallizing the sulfinic acid. The precipitation of sulfate as  $\text{BaSO}_4$  especially was found to cause losses because of the sulfinic acid carried down with the precipitate (*cf.* also Simonsen (6)) from which, however, it can be extracted with  $\text{HCl}$ . The attempts to isolate both the  $\text{Ba}$  salt and the sulfinic acid are as follows.

$\text{Hg}$  was removed by  $\text{H}_2\text{S}$  from 21.8 gm. of the precipitate; the

filtrate after  $\text{H}_2\text{S}$  had been expelled was made slightly alkaline to litmus with  $\text{Ba}(\text{OH})_2$ ,  $\text{BaSO}_4$  was filtered off, and the filtrate aerated (0.1 cc. of 1 N  $\text{CuSO}_4$  was added) until the nitroprusside test became negative. The solution was then evaporated, taken up in a small amount of  $\text{H}_2\text{O}$ , filtered from cystine, and the Ba salt of sulfinic acid precipitated by 4 volumes of alcohol; yield 1.3 gm. This material contained 29.3 per cent Ba; 219 gm. consumed 1 mm of  $\text{I}_2$  (equivalent to 1 gm. atom of O) on oxidation to cysteic acid; 222 gm. liberated 1 mm of  $\text{I}_2$  on KI-HCl reduction. ( $\text{R}-\text{SO}_2$ ) $_2\text{Ba}$ , molecular weight 441.6, contains 31.1 per cent Ba, consumes 1 mm of  $\text{I}_2$  per 220.8 gm., and liberates 1 mm of  $\text{I}_2$  per 147.2 gm.

In another case Hg was removed as  $\text{HgS}$  from 18.3 gm. of the precipitate; the filtrate (700 cc.) contained 8.5 mm of acid derivatives in addition to  $\text{H}_2\text{SO}_4$ , 6.5 mm of  $\text{R}-\text{SO}_2\text{H}$  (KI-HCl reduction value), and 23.6 mm of  $\text{R}-\text{SH}$ . After the solution was neutralized to the turning point of litmus with  $\text{Ba}(\text{OH})_2$ , the  $\text{BaSO}_4$  was filtered off and the filtrate aerated and evaporated at approximately  $30^\circ$ , taken up in a small amount of  $\text{H}_2\text{O}$  (6 days after starting), and filtered from cystine. The yellow filtrate (50 cc.) contained 4.3 mm of  $\text{R}-\text{SO}_2\text{H}$  (an additional 0.63 mm was extracted from the  $\text{BaSO}_4$  by 25 cc. of 2 N HCl). A slight excess of  $\text{H}_2\text{SO}_4$  was added, the solution heated with charcoal, filtered from both  $\text{BaSO}_4$  and charcoal, and evaporated. Since crystallization could not be induced, the syrup produced by evaporation was repeatedly heated with acetone and decanted until a solid was obtained; yield 0.97 gm. This material after correction for the 5.3 per cent cystine which was present possessed an equivalent weight of 171.5 based on titration with alkali (bromthymol blue as indicator). This corresponds to both cysteic acid (mol. wt. 169) and  $\text{R}-\text{SO}_2\text{H}\cdot\text{H}_2\text{O}$  (mol. wt. 171). The KI-HCl reduction indicated that the substance contained 50.3 per cent  $\text{R}-\text{SO}_2\text{H}\cdot\text{H}_2\text{O}$ , and the  $\text{I}_2$  oxidation showed 77 per cent, the remainder in each case being presumably cysteic acid. It appears that cysteic acid formation does take place to a certain extent, possibly during aeration, although this has not been confirmed as yet.

A method which offers promise consists of triturating a thin suspension of the precipitate with  $\text{BaCl}_2$ . Because of differences



in solubility in chloride solutions, the sulfinic acid is thereby separated at once from cysteine. However, the details of obtaining a solid material by this procedure have yet to be worked out.

*Behavior of Cystine Derivatives with  $\text{HgCl}_2$ —Mercuric chloride*

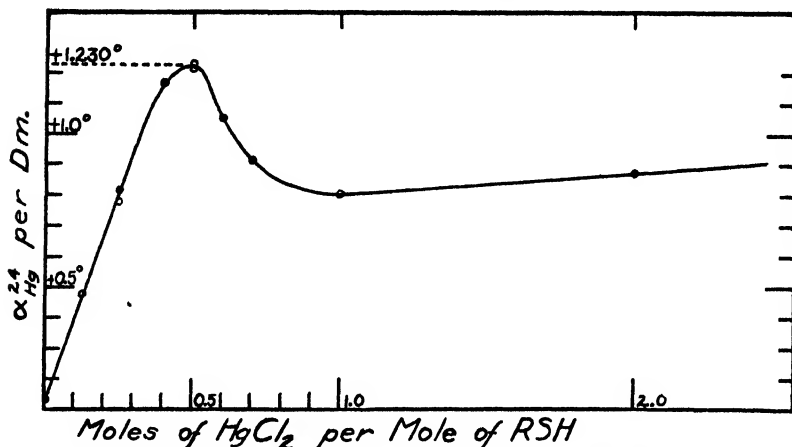


FIG. 2. Change in rotation of solutions containing 1 mM of cysteine in 10 cc. of  $\text{M}$   $\text{HCl}$  with the amount of  $\text{HgCl}_2$  present. Several precipitates were isolated and suspended in  $\text{H}_2\text{O}$ ,  $\text{Hg}$  determined as  $\text{HgS}$ ,  $\text{R-SH}$  determined colorimetrically after  $\text{H}_2\text{S}$  had been expelled;  $\text{HCl}$  was titrated with  $\text{NaOH}$ .

| Ratio, $\text{R-SH}:\text{HgCl}_2$<br>in solution | Analysis of ppt. |               |               |
|---|------------------|---------------|---------------|
|   | $\text{Hg}^{++}$ | $\text{R-SH}$ | $\text{Cl}^-$ |
|   | $\text{M}$       | $\text{M}$    | equivalents   |
| 1:0.5*  | 1.00             | 1.93          | 1.008         |
| 1:3   | 3.00             | 2.00          | 4.92          |
| 1:4†  | 1.98             | 1.00          | 4.90          |

\* The precipitate loses crystalline structure on addition of  $\text{H}_2\text{O}$ .

† This precipitate was washed with alcohol and ether.

because of its un-ionized nature, differs materially from the sulfate in its behavior with cystine and its derivatives. Although dismutation of cystine slowly takes place with higher ratios of  $\text{HgCl}_2:\text{R-S-S-R}$ , nevertheless the optical rotation of cystine in  $\text{M}$   $\text{HCl}$  (1 gm. per 100 cc.) is unchanged by the addition of 2  $\text{M}$

equivalents of  $\text{HgCl}_2$  ( $\alpha_{\text{Hg}}^{26} = -2.50^\circ$  per dm.) and remains constant for at least 3 days.

Cysteic acid behaved similarly;  $\alpha_{\text{Hg}}^{27}$  of a solution of 1 gm. of  $\text{R-SO}_2\text{H}$  in 100 cc. of  $\text{M HCl}$  was  $+0.116^\circ$  per dm., which remained unchanged with ratios of  $\text{HgCl}_2$  to cysteic acid of 0.5:1 and 3:1 ( $+0.115^\circ$  and  $+0.117^\circ$  per dm. respectively).

The rotation,  $\alpha_{\text{Hg}}^{26}$ , of 1 gm. of  $\text{R-SO}_2\text{H}$  in 100 cc. of  $\text{M HCl}$  amounted to  $+0.293^\circ$  per dm., which was unchanged by the addition of 0.5  $\text{M}$  equivalents of  $\text{HgCl}_2$ ; with a ratio of  $4\text{HgCl}_2$  to  $1\text{R-SO}_2\text{H}$ , the rotation was  $+0.306^\circ$  per dm.

With cysteine the rotation varied with the amount of  $\text{HgCl}_2$  present through a maximum when the solution contained  $1\text{R-SH}$  to  $0.5\text{HgCl}_2$  and reached a minimum value when  $1\text{R-SH}:1\text{HgCl}_2$  was present as shown by Fig. 2. Moreover, the compounds responsible for the changes in rotation evidently possess different solubilities, since precipitation in the form of loose needles starts with a ratio of  $1\text{R-SH}:0.25\text{HgCl}_2$  and continues through 1:0.7 (denser precipitate). No precipitation occurred with 1:1 and 1:2 ratios of cysteine to  $\text{HgCl}_2$  but with a 1:3 ratio a densely granular precipitate is formed which comes out so quickly at 1:4 that the rotation could not be taken.

Evidently the anomalously high rotation of the cysteine-Ag complex first noted by Vickery and Leavenworth (7) is a general characteristic of all cysteine-metal compounds.

#### DISCUSSION

The foregoing evidence may be accepted as indicating that the products of the dismutation of cystine are cysteine and an intermediate oxidation product, the sulfinic acid, rather than cysteine and cysteic acid. Although more definite proof of the presence of the sulfinic acid is desirable, nevertheless the material presented warrants further consideration of the dismutation problem. Of the published data, Vickery and Leavenworth (7) report 72 to 84 per cent recovery of cystine (based on N determinations after removal of Ag), figures which may be high owing to the presence of the sulfinic acid; of the remainder of the cystine, 0.3 to 1.7 per cent was isolated as cysteic acid, while the balance was presumably in an unidentified syrup. Simonsen (8) consistently found that about 75 per cent of the cystine was converted to cysteine by

Hg dismutation and it should be pointed out that the analytical data (N, S, and acid value) reported by her for the isolated cysteic acid could also indicate a monohydrate of the sulfinic acid,  $R-SO_2H \cdot H_2O$ , as isolated by Schubert (9). In regard to the quantitative results obtained by Preisler and Preisler (10) for the dismutation of dithiodihydracrylic acid, it can only be said that, while this compound evidently dismutates to form a sulfonic acid, the analogy with cystine is unwarranted.

Because of the lack of success attending the attempts at isolation of the sulfinic acid it cannot be unequivocally asserted that the sulfinic acid is the intermediate oxidation product produced, although the indirect evidence is most pointed. One fact which should perhaps be considered is the difficulty experienced in removing  $H_2S$  after precipitating Hg from both sulfinic acid solutions and solutions of dismuted cystine, which suggests interaction of  $H_2S$  with a cystine derivative and its subsequent slow elimination.<sup>4</sup> In fact the capabilities of  $H_2S$  for reacting with other sulfur compounds as well as its reducing properties should advise caution in its use. Similarly the use of heavy metal salts in studying the dismutation suffers from the disadvantage that they are capable of several valence states and may participate in the oxidation and reduction rather than acting merely as precipitating agents. Thus, the formation of  $Hg^+$  (first noted by Preisler and Preisler (10)) and its part in the dismutation is still unexplained.

#### SUMMARY

Cysteine, cysteic acid, and the sulfinic acid of cysteine ( $R-SO_2H$ ) are all precipitated from 2 N  $H_2SO_4$  solution by  $HgSO_4$ . Analytical evidence indicates that the precipitate obtained from cystine and  $HgSO_4$  contains cysteine and sulfinic acid; consequently, the equation  $2R-S-S-R + 2H_2O \rightarrow 3R-SH + R-SO_2H$  governs the dismutation of cystine rather than the previously proposed equation,  $3R-S-S-R + 3H_2O \rightarrow 5R-SH + R-SO_2H$ . This conclusion is supported by the amount of cysteine produced and by the evidence for the sulfinic acid, which

<sup>4</sup> Since sulfinic acids are known to react with free sulfur (11) ( $R-SO_2H + S \rightarrow R-SO_2SH$ ), the slow elimination of  $H_2S$  as well as cysteic acid formation could be explained by hydrolysis ( $R-SO_2 \cdot SH + H_2O \rightarrow H_2S + R-SO_2H$ ).

rests mainly on its power of liberating  $I_2$  from KI-HCl solutions; the isolation of a pure sulfinic acid was not accomplished. Reversal of the dismutation, i.e. reformation of cystine, occurs when the mercury is removed from the precipitate.

While  $HgCl_2$  does not affect the optical rotation of HCl solutions of cystine, sulfinic acid, or cysteic acid, the rotation of cysteine was found to depend on the amount of  $HgCl_2$  present.

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## CHEMICAL STUDIES ON THE VIRUS OF TOBACCO MOSAIC

### VIII. THE ISOLATION OF A CRYSTALLINE PROTEIN POSSESSING THE PROPERTIES OF AUCUBA MOSAIC VIRUS\*

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Aucuba mosaic virus may be regarded as a severe strain of tobacco mosaic virus. The two viruses are strikingly different, however, since the leaves of Turkish tobacco plants diseased with ordinary tobacco mosaic virus present a mottled or mosaic pattern of light and dark green areas, whereas those diseased with aucuba mosaic virus present a pattern of large, bright yellow areas surrounded by green or yellowish green areas (1). Furthermore, aucuba mosaic virus causes local lesions but no systemic infection in *Nicotiana sylvestris*, Spegaz. and Comes, whereas ordinary tobacco mosaic virus causes a systemic infection in this plant (2). McKinney (3) pointed out that plants diseased with tobacco mosaic virus often have small, bright yellow spots on the leaves and that the virus in these spots differs from that of ordinary tobacco mosaic. Jensen (4) was able to isolate many different yellow or aucuba-like mosaic viruses from plants infected with what he believed to be a single infectious unit of tobacco mosaic virus. Hence, he considers that the yellow mosaic viruses arise during the multiplication of tobacco mosaic virus in infected plants by some process apparently similar to that of mutation. There is evidence, therefore, that, in the plant, tobacco mosaic virus may give rise to aucuba or an aucuba-like mosaic virus. Further evidence for the existence of a close relationship between tobacco and aucuba mosaic has been obtained by serological

\* A preliminary report of this work was presented before the Society of American Bacteriologists (*J. Bact.*, 31, 52 (1936)).

methods (5). Since a crystalline protein possessing the properties of tobacco mosaic virus has been isolated from extracts of plants diseased with this virus (6), it seemed of importance to determine whether or not a crystalline protein could be isolated from plants diseased with the aucuba mosaic virus and to compare the properties of any protein isolated with those of the tobacco mosaic virus protein. The present paper records the isolation, from Turkish tobacco plants diseased with aucuba mosaic virus, of a crystalline protein, differing from the previously isolated tobacco mosaic virus protein, and possessing the properties of aucuba mosaic virus.

#### EXPERIMENTAL

*Preparation of Crystalline Protein*—The methods employed in the concentration, purification, crystallization, and testing of the aucuba mosaic virus protein were similar to those previously used for the crystalline tobacco mosaic virus protein. Since these methods have been described in detail (7), only a short summary giving the analytical data will be presented here.

About 100 kilos of mature Turkish tobacco plants diseased with aucuba mosaic virus were frozen and then put through a meat grinder. Sufficient of a concentrated solution of disodium phosphate to bring the phosphate concentration to 0.1 M was added with thorough stirring, and after the pulp had thawed the juice was pressed out. The extract of about 60 liters, which was at pH 7, contained 1.94 mg. of total nitrogen, and 0.75 mg. of protein nitrogen per cc. before filtration through celite (Hyflo Super-cel), and approximately the same amounts of nitrogen after filtration. The pulp was extracted with 45 liters of 0.1 M  $\text{Na}_2\text{HPO}_4$ . This extract was found to be at pH 7.2 and to contain 0.55 mg. of total nitrogen and 0.18 mg. of protein nitrogen per cc. before, and 0.37 mg. of total nitrogen and 0.15 mg. of protein nitrogen per cc. after filtration through celite. The two extracts, containing about 280 gm. of protein, were combined and the globulin fraction was precipitated by adding sufficient solid ammonium sulfate to bring the salt concentration to 30 per cent by weight. The globulin fraction was removed by filtration, taken into solution in 0.1 M phosphate at pH 7, filtered through celite, and precipitated two additional times to give 135 gm. of crude, very dark brown protein. This

three times precipitated protein was considerably darker in color than the usual preparations of twice precipitated tobacco mosaic protein. This may have been due to the fact that the plants were quite old when harvested. In an attempt to remove this color, the 135 gm. portion of protein was dissolved in 15 liters of water, the solution adjusted to pH 8.2, and the protein precipitated by the addition of 3 kilos of ammonium sulfate and removed by filtration, a layer of celite being used on a Buchner funnel. The protein was removed from the celite by extraction with water at pH 7.3. This procedure was repeated two additional times, when the filtrate from the celite filter cake was practically colorless. A solution of the protein in 24 liters of water, which was definitely yellow in color, was adjusted to pH 4.5, and 2 per cent by weight of celite added and the mixture filtered by means of a Buchner funnel. The active protein was retained on the celite filter cake and much color and a small amount of inactive protein were obtained in the filtrate. The active protein was removed from the celite by extraction with water at pH 8 to give a very opalescent, yellow-colored solution of active protein. The protein in this solution was crystallized by first adding sufficient amounts of a saturated solution of ammonium sulfate to give a slight turbidity and then adding slowly a solution of 5 per cent glacial acetic acid in 0.5 saturated ammonium sulfate until the hydrogen ion concentration was pH 4.5. Crystallization was completed by adding a saturated solution of ammonium sulfate until the total concentration of salt was about 10 per cent by weight. Crystallization may also be accomplished with considerably smaller amounts of ammonium sulfate. Very good crystals were obtained after two recrystallizations despite the fact that the solution of the protein was yellow-colored. Fig. 1 is a reproduction of a photomicrograph of the crystals which were obtained. The crystals are needles about 0.03 mm. long. Under similar conditions of crystallization, the crystals of the aucuba mosaic protein are somewhat larger than those of the tobacco mosaic protein. An identical or similar crystallizable, active protein has been isolated from extracts of tomato plants diseased with aucuba mosaic virus.

*General Properties of Crystalline Protein*—A solution containing 1 mg. per cc. of aucuba mosaic protein gives a positive test with Millon's, biuret, xanthoproteic, glyoxylic acid, and Folin's tyro-



sine reagents. The Molisch test is negative, even with concentrated solutions or after alkaline hydrolysis. However, after standing for about 10 hours, a faint ring of violet color develops in the reaction mixture. The material is precipitated by the usual protein-precipitating agents such as trichloroacetic acid, phosphotungstic acid, safranine, tannic acid, alcohol, and ammonium or magnesium sulfate. When solutions of the protein are made more alkaline than about pH 11 or more acid than about pH 1, or are heated to about 70°, the protein is denatured and the virus activity is lost. Under comparable conditions, solutions of the aucuba mosaic protein possess a more silky and opalescent appearance than do those of the tobacco mosaic protein. Wyckoff and



FIG. 1. Reproduction of a photograph of crystalline aucuba mosaic virus protein isolated from infected Turkish tobacco plants.  $\times 393$ . Photograph by J. A. Carlile

Corey (8) determined the x-ray diffraction pattern of the crystalline aucuba mosaic protein and found it to be the same as that of tobacco mosaic protein. The decided opalescent appearance of solutions of the protein disappears when such solutions are made more alkaline than about pH 11. The elementary analysis of samples of aucuba mosaic protein prepared for analysis by two different methods is given in Table I. The Cl, P, S, and ash values have been found to vary considerably depending on the method of preparation of the samples for analysis and on the previous history of the protein. It may be seen that precipitation of the protein with trichloroacetic acid caused a large increase in the Cl content. Larger Cl, S, and ash values were obtained

when the protein was subjected to only a short dialysis. Protein prepared and dialyzed at hydrogen ion concentrations between pH 3 and 7 was found to have nucleic acid<sup>1</sup> associated with it. The delayed, weak Molisch reaction previously noted is probably due, not to an impurity, but to the carbohydrate, probably *d*-ribose, released on acid hydrolysis of the nucleic acid. The nucleic acid content of various preparations of tobacco mosaic virus protein, determined after a 2 hour hydrolysis at 5° with 5 per cent sodium hydroxide, was found to vary between about

TABLE I

*Elementary Analysis and Optical Activity of Crystalline Aucuba Mosaic Virus Protein*

Samples were dialyzed against water at pH 7 for 1 day and then against dilute HCl at pH 3.7 for 4 days. Sample 1 was precipitated with acetone and dried over P<sub>2</sub>O<sub>5</sub> *in vacuo*, at 60°. Sample 2 was precipitated with hot 2.5 per cent trichloroacetic acid, washed eight times with cold 0.5 per cent trichloroacetic acid, then twice with water, and dried over P<sub>2</sub>O<sub>5</sub> *in vacuo*, at 60°. The fourth and subsequent wash liquids gave no test for sulfate. Solutions containing about 10 mg. of protein per cc. at pH 11.4 ± 0.1 were used for the optical activity determinations.

| Sample No.  | Per cent of ash-free dry weight |      |       |      |      |      | Ash  |
|---|---------------------------------|------|-------|------|------|------|--|
|   | C                               | H    | N     | Cl   | P    | S    |  |
| 1   | 50.46                           | 6.88 | 16.52 | 0.14 | 0.51 | 0.24 | 0.11   |
|   | 50.30                           | 6.94 | 16.59 | 0.12 |      |      | 0.17   |
| 2   | 49.07                           | 6.64 | 16.67 | 1.35 |      |      | 0.19   |
|   | 49.33                           | 6.68 | 16.79 | 1.36 |      |      | 0.11   |
| Optical activity, $[\alpha]_D^{25}$ per mg. nitrogen..... |                                 |      |       |      |      |      | $\begin{cases} 0.41^\circ \\ 0.43^\circ \end{cases}$ |

1.7 and 3.4 per cent, depending on the previous history of the protein. It was found possible to remove the nucleic acid and to obtain phosphorus-free protein possessing virus activity by dialysis at pH 8 or 9, hence the phosphorus appears to be located solely in the nucleic acid associated with the virus protein. As may be seen from Table I, the optical activity of aucuba mosaic protein is the same as that of tobacco mosaic protein. The opalescence of solutions of ordinary tobacco and aucuba mosaic

<sup>1</sup> The presence of nucleic acid in certain preparations of tobacco mosaic virus protein was called to the writer's attention by Dr. N. W. Pirie.

virus proteins made it impossible to determine the optical activity of such solutions at hydrogen ion concentrations at which the protein was still active. It was necessary to make the solutions sufficiently alkaline to cause the disappearance of the opalescence in order to obtain solutions which could be used in the polariscope. It should be noted, therefore, that the rotations, not of solutions of intact virus protein molecules, but of solutions of inactive proteins were measured. The rotations of such solutions, determined

TABLE II

*Comparison of Activities of Tobacco and Aucuba Mosaic Virus Proteins*

| Test plant       | Virus protein | Dilution*         |                   |                   |                   |                   |                   |                   |
|------------------|---------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
|                  |               | 1:10 <sup>3</sup> | 1:10 <sup>4</sup> | 1:10 <sup>5</sup> | 1:10 <sup>6</sup> | 1:10 <sup>7</sup> | 1:10 <sup>8</sup> | 1:10 <sup>9</sup> |
| <i>Nicotiana</i> | Tobacco       | 34.2†             | 69.8              | 17.9              | 6.2               | 5.3               | 6.9               | 4.2               |
| <i>glutinosa</i> | Aucuba        | 41.6‡             | 95.5              | 26.6              | 9.6               | 6.7               | 6.1               | 2.8               |
| <i>Phaseolus</i> | Tobacco       | 74.5†             | 24.7              | 14.0              | 1.7               | 2.2               | 1.0               | 1.3               |
| <i>vulgaris</i>  | Aucuba        | 97.5‡             | 45.4              | 25.0              | 4.8               | 1.1               | 0.2               | 0.8               |

\* Dilutions were made with 0.1 M phosphate buffer at pH 7. A dilution of 1:10<sup>3</sup> denotes 0.001 gm. of protein per cc., 1:10<sup>4</sup> denotes 0.0001 gm. of protein per cc., etc.

† Numbers represent the average number of lesions per half leaf obtained on ten or more half leaves of *Phaseolus vulgaris* or of *Nicotiana glutinosa* on inoculation with the designated preparation and dilution.

‡ Numbers represent the average number of lesions per half leaf obtained on the other halves of the same leaves on inoculation with the designated preparation and dilution. The halves of the leaves to which a given protein was applied were reversed at each dilution. For example, at a dilution of 1:10<sup>3</sup> aucuba protein was put on the right halves and tobacco protein on the left halves of the same leaves, while at a dilution of 1:10<sup>4</sup> aucuba protein was put on the left halves and tobacco protein on the right halves of the same leaves.

immediately after adjustment of the solutions to the alkaline reaction, did not change on standing for 24 hours.

The sera of animals injected with the juice or crystalline protein from plants diseased with tobacco mosaic or aucuba mosaic virus give a precipitate when mixed with a solution containing but 10<sup>-5</sup> gm. per cc. of the crystalline aucuba mosaic protein. As may be seen from Table II, the virus activity of aucuba mosaic protein and of tobacco mosaic protein is approximately the same when compared by the half leaf method (9). The question as to

whether or not the two proteins possess exactly the same virus activity will be considered in a subsequent paragraph. It should be pointed out that the dilutions given for the virus activity and the precipitin test in the case of the aucuba protein and those previously given for tobacco mosaic protein do not represent end-point titrations, but simply dilutions at which the tests are readily reproducible. Under favorable conditions, solutions containing but  $10^{-12}$  to  $10^{-14}$  gm. per cc. of the proteins may be demonstrated to possess virus activity, and solutions containing slightly less than  $10^{-8}$  gm. per cc. of the proteins may give the precipitin test. It should be emphasized that at such high dilutions the positive activity determinations, particularly, are not reproducible at will.

The virus activity is lost on treatment with oxidizing agents such as chloramine T, potassium permanganate, and hydrogen peroxide, or on treatment with formaldehyde or nitrous acid. The first two reagents cause denaturation of the protein and loss of ability to crystallize and to react with specific antisera. Hydrogen peroxide, formaldehyde, and nitrous acid, however, have the unusual action, first found in the case of tobacco mosaic virus protein (10), of causing loss of virus activity and yet altering the protein so slightly that the reaction products remain native and retain many of the characteristic chemical and serological properties of the aucuba mosaic virus protein.

*Distinguishing Properties of Crystalline Protein*—With the exception of the slightly larger crystals and the more pronounced silky, shimmering, opalescent appearance of the solutions, the properties of the aucuba mosaic virus protein which have been described are the same as those of tobacco mosaic virus protein. However, since their biological properties are so strikingly different, it seemed probable that some other properties of the two proteins might be different. This proved to be the case, for the solubility, isoelectric point, and the sedimentation constant of aucuba mosaic protein were found to be quite different from those of the tobacco mosaic protein. The pronounced opalescence of solutions of aucuba mosaic protein suggested that this protein might be less soluble than tobacco mosaic protein. Considerable trouble was encountered in solubility experiments with the proteins owing to great difficulty in the separation of protein in solution from that not in solution. However, sufficient data were secured

to demonstrate that aucuba mosaic protein is much less soluble than tobacco mosaic protein, and also that the two proteins probably form solid solutions. The samples used for the solubility determinations were prepared by adding to dialyzed solutions of

TABLE III

*Solubility Experiments with Crystalline Tobacco Mosaic and Aucuba Mosaic Virus Proteins*

|  |  |
|--|--|
| 10 cc. dialyzed preparation containing 13.2 mg. tobacco mosaic virus protein per cc. + 1.32 cc. 1 M $\text{PO}_4$ at pH 7 + 1.9 cc. $\text{H}_2\text{O}$ + 0.79 gm. $(\text{NH}_4)_2\text{SO}_4$ to give opalescent solution (A) | 10 cc. dialyzed preparation containing 13.8 mg. aucuba mosaic virus protein per cc. + 1.38 cc. 1 M $\text{PO}_4$ at pH 7 + 2.4 cc. $\text{H}_2\text{O}$ + 0.83 gm. $(\text{NH}_4)_2\text{SO}_4$ to give suspension of crystals (B) |
| Preparations A and B centrifuged 1 hr.   |  |
| Supernatant liquid from (A) contained 7.4 mg. protein per cc.  | Supernatant liquid from (B) contained 4.9 mg. protein per cc.  |
| Half of supernatant liquid of (A) + ppt. from (B) mixed for 0.5 hr. (C)  | Half of supernatant liquid of (B) + ppt. from (A) mixed for 0.5 hr. (D)  |
| Preparations C and D centrifuged 1 hr.   |  |
| Supernatant liquid from (C) contained 3.4 mg. protein per cc.  | Supernatant liquid from (D) contained 3.4 mg. protein per cc.  |
| Half of supernatant liquid of (A) centrifuged an additional 1 hr., after which it contained 6.0 mg. protein per cc. (Ppt. E)   | Half of supernatant liquid of (B) centrifuged an additional 1 hr., after which it contained 1.9 mg. protein per cc. (Ppt. F)   |
| Preparations E and F stirred separately for 0.5 hr. with 2 cc. each of solvent of same composition as used for Preparations A and B; then centrifuged 1 hr.  |  |
| Supernatant liquid from (E) + solvent contained 4.0 mg. protein per cc.  | Supernatant liquid from (F) + solvent contained 1.1 mg. protein per cc.  |

the two proteins sufficient amounts of 1 M phosphate buffer, water, and solid ammonium sulfate, so that each solution contained 10 mg. of protein and equal amounts of phosphate buffer and ammonium sulfate per cc. The experimental details are recorded in Table III. It may be seen that the tobacco mosaic protein is more

soluble than the aucuba mosaic protein whether the solubility be determined from the supersaturated or the undersaturated side. The difficulties in obtaining solubility data are evident, since on centrifugation of the same preparation of tobacco mosaic protein for successive 1 hour intervals, the protein figure dropped from 10 mg. to 7.4 mg. to 6.0 mg. per cc., whereas in the case of the aucuba mosaic protein the decrease was from 10 mg. to 4.9 mg. to 1.9 mg. per cc. Despite these difficulties, centrifugation was found to be much more satisfactory than filtration for the separation of protein not in solution from that in solution. The fact that nearly the same solubility was obtained in each case from the supersaturated and from the undersaturated side is an indication that the true solubilities of the proteins were being measured. Although duplicate or successive solubility determinations on separate portions of the same protein preparation have always given the same value, it should be mentioned that considerable variation has been found in the solubility of aucuba mosaic protein prepared at different times from different lots of plants. It may also be seen from Table III that mixtures of the two proteins prepared by dissolving aucuba mosaic protein in a saturated solution of tobacco mosaic protein, or by dissolving tobacco mosaic protein in a saturated solution of aucuba mosaic protein have the same solubility. The fact that the solubility of such mixtures lies between the solubilities of the two separate proteins indicates that they form solid solutions. The solubility experiments indicate that the two proteins are probably different although closely related proteins.

The isoelectric point of aucuba mosaic protein was determined by Dr. Loring and found to be pH 3.7, which is 0.4 pH unit more alkaline than the isoelectric point of tobacco mosaic protein when determined under the same conditions. An even more striking difference was demonstrated when Dr. Wyckoff and Mr. Biscoe, who determined the sedimentation constants, found that of the aucuba protein to be about 20 per cent greater than that of tobacco mosaic protein prepared under comparable conditions (11). If they have the same general shape the molecules are, therefore, larger than those of tobacco mosaic protein. This would be in accord with the lower solubility of the aucuba mosaic protein. Previous ultrafiltration experiments (12) indicated that aucuba mosaic virus was about 3 times larger than ordinary tobacco

mosaic virus. The filtration and ultracentrifugal experiments are in general accord, except that the latter indicate that the difference in size is considerably less than a 3:1 ratio.

*Preparation of Partially Active Protein*—In an attempt to remove the yellow color from the solution of aucuba mosaic protein, a sample was repeatedly processed during the course of 3 weeks by

TABLE IV

*Effect of Prolonged Fractionation on Activity of Aucuba Mosaic Protein. Activity Compared with That of Tobacco Mosaic Protein Used in Tests Given in Table II*

| Conditions                                 | Test plant                 | Protein | Dilution* of tobacco protein |                   |                   |
|--|----------------------------|---------|------------------------------|-------------------|-------------------|
|  |                            |         | 1:10 <sup>3</sup>            | 1:10 <sup>4</sup> | 1:10 <sup>5</sup> |
| Two proteins at same concentration         | <i>Phaseolus vulgaris</i>  | Tobacco | 15.7†                        | 7.7               | 3.3               |
|  |                            | Aucuba  | 10.8‡                        | 5.1               | 1.8               |
|  | <i>Nicotiana glutinosa</i> | Tobacco | 38.1†                        | 30.6              | 8.0               |
|  |                            | Aucuba  | 23.4‡                        | 17.4              | 5.4               |
| 2 times as much aucuba as tobacco protein  | <i>Phaseolus vulgaris</i>  | Tobacco | 98.5                         | 55.0              | 15.4              |
|  |                            | Aucuba  | 50.0                         | 18.7              | 9.3               |
| 10 times as much aucuba as tobacco protein | <i>Phaseolus vulgaris</i>  | Tobacco | 63.0                         | 42.6              | 20.6              |
|  |                            | Aucuba  | 47.5                         | 36.0              | 23.8              |

\* Dilutions were made with 0.1 M phosphate buffer at pH 7. A dilution of 1:10<sup>3</sup> denotes 0.001 gm. of protein per cc., 1:10<sup>4</sup> denotes 0.0001 gm. of protein per cc., etc.

† Numbers represent the average number of lesions per half leaf obtained on ten or more half leaves of *Phaseolus vulgaris* or of *Nicotiana glutinosa* on inoculation with the designated preparation and dilution.

‡ Numbers represent the average number of lesions per half leaf obtained on the other halves of the same leaves on inoculation with the designated preparation and dilution. The halves of the leaves to which a given protein was applied were reversed at each dilution. For example, at a dilution of 1:10<sup>3</sup> aucuba protein was put on the right halves and tobacco protein on the left halves of the same leaves, while at a dilution of 1:10<sup>4</sup> aucuba protein was put on the left halves and tobacco protein on the right halves of the same leaves.

treatment with 10 per cent and 20 per cent ammonium sulfate and celite at pH 8, by adsorption on celite at pH 4.5 and elution at pH 8, and by treatment with calcium oxide. During the course of this treatment, in which the amount of the protein was decreased by about 90 per cent, the remaining protein appeared to become more and more insoluble. The silky and opalescent appearance

of the protein solution gradually increased until even very dilute solutions of the protein had the characteristic silky appearance when stirred. Despite the fact that the protein appeared to be out of solution, centrifugation for 30 minutes at 3000 R.P.M. did not cause the protein to sediment to the bottom of the tube. The virus activity of this protein was determined and, as may be seen in Table IV, it was found to be only about 10 per cent as active as the tobacco mosaic protein with which it was compared. This is the same sample of tobacco mosaic protein with which it was originally compared (see Table II), hence the aucuba mosaic protein has been about 90 per cent inactivated. This phenomenon is the same as that which was encountered when tobacco mosaic protein was repeatedly processed over a long period of time at room temperature. The virus protein has been found to be very sensitive towards even mild oxidizing agents, such as hydrogen peroxide, and it seems possible that the increase in the silky and opalescent appearance of the solutions and the loss of activity, which take place on long continued treatment of the protein at room temperature, may result from oxidation.

*Comparison of Virus Activity of Aucuba Mosaic and Tobacco Mosaic Protein*—The ultracentrifugal data (11) indicate that the molecules of aucuba mosaic protein are probably larger than those of tobacco mosaic protein, hence the former should possess a lower activity than the latter, on a weight for weight basis, if other factors are the same. However, since the difference is of the order of 20 per cent, a given weight of tobacco protein would not be expected to be over 20 per cent more infectious than the same amount of aucuba protein. Although this difference is not much greater than that which may be detected with a moderate number of plants, it was decided to make a careful comparison of the activities of the two preparations. It seemed desirable, in order to eliminate many chances for error, to prepare the two protein samples under as nearly comparable conditions as possible. Accordingly, a group of young Turkish tobacco plants was divided into two comparable groups. One group was inoculated with a solution of 2 mg. of aucuba mosaic protein and the other group was inoculated with a similar amount of tobacco mosaic protein. The plants were cut 4 weeks later and processed according to the improved method for the preparation of crystalline protein (7)



under as nearly identical conditions as possible. This and other subsequently prepared samples of aucuba mosaic protein were found to give practically colorless solutions. The activities of the

TABLE V

*Comparison of Activities of Tobacco and Aucuba Mosaic Protein Preparations Prepared under Comparable Conditions*

(a) First phosphate extracts diluted without regard to protein content, (b) same after dilution so that both contain same amount of protein, *viz.*, on protein basis, and (c) preparations on protein basis following purification but before crystallization of the proteins.

| Preparation | Test plant                 | Protein | Dilution*         |                   |                   |                   |          |           |
|-------------|----------------------------|---------|-------------------|-------------------|-------------------|-------------------|----------|-----------|
|             |                            |         | Undiluted         | 1:10              | 1:100             | 1:1000            | 1:10,000 | 1:100,000 |
| (a)         | <i>Phaseolus vulgaris</i>  | Tobacco | 27.0†             | 58.3              | 125.0             | 63.2              | 10.7     | 4.0       |
|             |                            | Aucuba  | 23.0‡             | 78.2              | 78.2              | 31.6              | 4.6      | 0.5       |
|             |                            |         | 1:10 <sup>3</sup> | 1:10 <sup>4</sup> | 1:10 <sup>5</sup> | 1:10 <sup>6</sup> |          |           |
| (b)         | <i>Nicotiana glutinosa</i> | Tobacco | 54.5†             | 23.4              | 13.3              | 1.5               |          |           |
|             |                            | Aucuba  | 50.6‡             | 21.5              | 15.7              | 4.2               |          |           |
| (c)         | <i>Phaseolus vulgaris</i>  | Tobacco | 102.0             | 78.5              | 86.9              | 5.0               |          |           |
|             |                            | Aucuba  | 105.0             | 72.8              | 53.6              | 11.7              |          |           |
|             | <i>Nicotiana glutinosa</i> | Tobacco | 28.1              | 24.8              | 11.2              | 1.6               |          |           |
|             |                            | Aucuba  | 25.6              | 38.1              | 11.5              | 3.0               |          |           |

\* Dilutions were made with 0.1 M phosphate buffer at pH 7. A dilution of 1:10<sup>3</sup> denotes 0.001 gm. of protein per cc., 1:10<sup>4</sup> denotes 0.0001 gm. of protein per cc., etc.

† Numbers represent the average number of lesions per half leaf obtained on ten or more half leaves of *Phaseolus vulgaris* or of *Nicotiana glutinosa* on inoculation with the designated preparation and dilution.

‡ Numbers represent the average number of lesions per half leaf obtained on the other halves of the same leaves on inoculation with the designated preparation and dilution. The halves of the leaves to which a given protein was applied were reversed at each dilution. For example, at a dilution of 1:10<sup>3</sup> aucuba protein was put on the right halves and tobacco protein on the left halves of the same leaves, while at a dilution of 1:10<sup>4</sup> aucuba protein was put on the left halves and tobacco protein on the right halves of the same leaves.

first disodium phosphate extracts, before and following dilution so that both preparations contained the same amount of protein, and the activities of the protein solutions, after the CaO treatment but before crystallization of the protein, were compared by the half

leaf method. The latter samples were diluted with 0.1 M phosphate at pH 7 so that they contained the same amount of protein per cc. before they were used for activity tests. The subsequent dilutions were also made with 0.1 M phosphate at pH 7. The results, which are given in Table V, show that the extract from

TABLE VI

*Comparison of Activities of Crystalline Aucuba and Crystalline Tobacco Mosaic Virus Proteins Prepared under Comparable Conditions*

| Test No. | Test plant                 | Protein | Dilution*         |                   |                   |                   |                   |
|----------|----------------------------|---------|-------------------|-------------------|-------------------|-------------------|-------------------|
|          |                            |         | 1:10 <sup>3</sup> | 1:10 <sup>4</sup> | 1:10 <sup>5</sup> | 1:10 <sup>6</sup> | 1:10 <sup>7</sup> |
| 1        | <i>Phaseolus vulgaris</i>  | Tobacco | 127.0†            | 68.0              | 29.8              | 5.7               | 0.4               |
|          |                            | Aucuba  | 140.8†            | 86.5              | 23.8              | 4.7               | 0.3               |
|          | <i>Nicotiana glutinosa</i> | Tobacco | 63.5†             | 25.9              | 9.9               | 2.9               | 0.4               |
|          |                            | Aucuba  | 58.0†             | 41.2              | 16.7              | 3.0               | 0.3               |
| 2        | <i>Phaseolus vulgaris</i>  | Tobacco | 97.1              | 94.0              | 30.8              | 5.1               | 0.2               |
|          |                            | Aucuba  | 54.0              | 32.1              | 21.0              | 9.2               | 0.9               |
|          | <i>Nicotiana glutinosa</i> | Tobacco | 121.2             | 42.5              | 12.8              | 1.4               | 0.8               |
|          |                            | Aucuba  | 120.0             | 68.5              | 14.6              | 1.0               | 0.2               |
| 3        | <i>Phaseolus vulgaris</i>  | Tobacco |                   | 88.0              | 51.2              | 13.2              |                   |
|          |                            | Aucuba  |                   | 97.0              | 49.0              | 8.8               |                   |
| 4        | " "                        | Tobacco |                   | 103.7             | 65.4              | 14.7              |                   |
|          |                            | Aucuba  |                   | 101.5             | 64.1              | 28.0              |                   |
| 5        | " "                        | Tobacco |                   | 71.8              | 40.0              | 16.8              |                   |
|          |                            | Aucuba  |                   | 55.6              | 37.3              | 17.2              |                   |

\* Dilutions were made with 0.1 M phosphate buffer at pH 7. A dilution of 1:10<sup>3</sup> denotes 0.001 gm. of protein per cc., 1:10<sup>4</sup> denotes 0.0001 gm. of protein per cc., etc.

† Numbers represent the average number of lesions per half leaf obtained on twenty or more half leaves of *Phaseolus vulgaris* or of *Nicotiana glutinosa*, ten or more of which were inoculated on the right halves and ten or more of which were inoculated on the left sides with the designated preparation and dilution.

‡ Numbers represent the average number of lesions per half leaf obtained on the other halves of the leaves referred to in the preceding foot-note on inoculation with the designated preparation and dilution.

tobacco mosaic-diseased plants is more active than the extract from aucuba mosaic-diseased plants. Since the extract of Turkish tobacco plants diseased with aucuba mosaic virus contains less protein than the extract of similar plants diseased with tobacco mosaic virus, the former would be expected to be less active than

the latter. The results also show that, on a protein basis, the two extracts and the two purified protein preparations had about the same activity.

The activities of the crystalline preparations were compared in a series of tests using the half leaf method with *Nicotiana glutinosa*, L., and *Phaseolus vulgaris*, L., var. Early Golden Cluster, in order to determine whether or not the difference in the probable molecular weights of the two proteins causes a difference in the activities. It may be seen from Table VI that, on a weight for weight basis, no significant difference was found between the activity of the tobacco mosaic virus protein and that of the aucuba mosaic virus protein used in the present tests. Although the activities are certainly of the same order, it is possible that there may be an actual difference in activity. It seems preferable to reserve final judgment until the molecular weights are known with greater certainty and until the activities of proteins, prepared under similar conditions and having the greatest possible difference in molecular weights, are compared.

#### DISCUSSION

The isolation, from Turkish tobacco plants diseased with aucuba mosaic virus, of a crystalline protein differing from the protein isolated from Turkish tobacco plants diseased with ordinary tobacco mosaic virus demonstrates that two different strains of a virus give rise to two different proteins. This is of prime importance, for it is an indication, at least, that viruses may be characterized as distinct proteins. It is problematical, of course, as to whether or not it may be possible to characterize all of the many strains of tobacco mosaic virus as separate and distinct proteins. It appears more likely that the strains of tobacco mosaic virus may consist of a family of closely related proteins, and that it may be possible to identify, chemically, a given protein only when its properties are appreciably different from those of the protein with which it is being compared. As a matter of fact, it is entirely possible that the crystalline tobacco mosaic and aucuba mosaic proteins already isolated may consist, in each case, not of single chemical individuals, but of groups of very closely related molecules, the different molecules of each group resembling each

other so closely as to be indistinguishable chemically, but the two groups differing so appreciably, as groups, that they may be distinguished chemically. The fact that many of the chemical and serological properties of ordinary tobacco mosaic virus protein and of aucuba mosaic virus protein are identical or very similar is an indication that they are closely related. In view of the evidence that ordinary tobacco mosaic virus may, in the plant, mutate or in some manner become altered so that new and different strains of virus are produced, it seems logical that the new virus protein so evolved should be fairly closely related to the original tobacco mosaic virus protein. The manner in which this change from a given virus protein to a different virus protein takes place is, of course, open to much speculation. Since the normal plant proteins do not possess high molecular weights (11) and do not possess properties similar to those of the high molecular weight virus proteins, the latter proteins appear to be produced, not by a simple rearrangement of a molecule of normal protein, but rather by polymerization or, more likely, by direct synthesis. If, during the synthesis by the plant of molecules of a given unusual, large protein, a single molecule containing an extra unit of structure or a slightly different arrangement of given structures should be produced, then such a molecule might theoretically cause the production of many more molecules of its own kind and thus give rise to the new virus strain. Under certain environmental conditions, such a new molecule might be more stable than the old, hence it is possible that environmental conditions during virus production may actually influence the production of such new molecules. For example, Holmes has secured evidence that mild or masked virus strains increase in plants held at temperatures too high for the increase of ordinary tobacco mosaic virus (13). It is conceivable that the masked virus is more stable in such plants and that there is an increased tendency for its production under such conditions. Furthermore Kunkel has obtained new and distinct strains of aucuba mosaic virus by holding young plants at a temperature of 35° for 3 days following infection with the ordinary strain of virus (2). The production of new strains is an extremely fascinating subject, but it is felt preferable to reserve further speculation until more experimental data are at hand.

## SUMMARY

A crystalline protein which has the properties of aucuba mosaic virus has been isolated from Turkish tobacco plants infected with this virus. The virus activity, chemical composition, optical rotation, crystalline appearance, x-ray diffraction pattern of crystals, and general chemical and serological properties of this protein are either identical or very similar to those of tobacco mosaic virus protein. Aucuba mosaic virus protein may be distinguished from the latter by its larger crystals, its more silky and opalescent solutions, its lower solubility, its more alkaline isoelectric point, and its greater sedimentation constant. The isolation, from Turkish tobacco plants diseased with aucuba mosaic virus, of a crystalline protein differing from tobacco mosaic virus protein, demonstrates that two different strains of a virus give rise to two different proteins.

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## THE EFFECT OF INHIBITORS ON SUCCINOXIDASE\*

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In a previous publication it was shown that succinic acid is rapidly oxidized by homogenized suspensions of chick kidney (1). In view of the fact that succinic acid is an important intermediate in tissue oxidations (2-4) it seemed desirable to study some of the properties of the succinoxidase system by means of the new technique.

Quastel and Woolridge (5), using the methylene blue technique, showed that toluene-treated *Bacillus coli* were able to oxidize succinic acid as easily as did the untreated organisms, although the treated organisms were inactive with respect to many other substrates. They demonstrated the fact that malonic acid markedly inhibits the oxidation of succinic acid. Later Quastel and Wheatley (6) included minced rabbit muscle and brain, and human brain in their studies, using the Barcroft technique. They used 0.5 gm. of minced tissue in a total volume of 3 cc. In the case of the animal tissues, malonic acid again inhibited the oxidation of succinic acid.

Clutterbuck (7) studied the conversion of succinic acid and fumaric acid into *l*-malic acid by a polarimetric method. He showed that 30 gm. of minced liver in a volume of about 500 cc. could convert 5 gm. of succinic acid to an equilibrium mixture of fumaric and malic acids (about 3.5 gm.) in 6 hours, while the conversion of fumaric acid required only 2 hours. It thus appears that both fumarase and succinoxidase were highly active at the dilution employed in his experiments, and that the fumarase reaction is the faster of the two. Ogston and Green (8) have

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concentrated succinic dehydrogenase from washed minced heart muscle, as well as from liver.

We have shown (1) that in dilute tissue suspensions the oxygen uptake in the absence of added substrate is greatly reduced, and that the addition of succinic acid allows a rapid oxygen uptake which is constant for as long as 2 hours when sufficient substrate is present. Under these conditions the oxidation of succinic acid can be studied with very little interference from other respiratory systems.

#### EXPERIMENTAL

The method of preparing the tissue and measuring the oxygen uptake has been previously described (1).

In the present study, tissues from both rats and chickens were studied. Since the oxygen uptake of a tissue suspension appears to be proportional to the concentration of tissue when an excess of succinic acid is present, it seemed reasonable that the oxygen uptake of a given amount of tissue, in the presence of succinic acid, would be a good measure of its succinoxidase content. On this basis, brain, liver, and kidney tissues from both rats and chickens were assayed for succinoxidase. The results are shown in Table I. In every case, 40 mg. of homogenized tissue were suspended in 1 cc. of medium, and, when succinate was added, the final concentration was 0.034 M, making 4 mg. of succinic acid per cc. Although this concentration of succinate is not sufficient to maintain a constant rate of oxygen uptake over a 2 hour period, it does give a quite uniform rate for 1 hour and was therefore used for the studies with inhibitors as well as the assays recorded in Table I.

There is a fairly close agreement between the values for the same type of tissue from both species of animals, although chick kidney is more active than rat kidney. The liver from both species contains less of the enzyme than the kidney but more than is found in brain.

A large number of experiments were carried out with various inhibitors, with chick kidney as a source of the enzyme, and a low concentration of tissue to eliminate other systems. Various investigators have reported that the succinoxidase system is poisoned by cyanide. Our preliminary experiments indicated but slight cyanide inhibition but this was soon traced to an error in

technique. Van Heyningen (9) showed that the apparent cyanide inhibition might be lowered owing to the fact that cyanide from the suspension medium is absorbed by the KOH in the CO<sub>2</sub> absorber, thus lowering the effective concentration of cyanide. He eliminated this error by adding cyanide to the KOH in the center cups with varying proportions of KCN and KOH "which are so adjusted as to have a concentration of free HCN equal to that in the outer cup" according to the suggestion of Krebs (10). We

TABLE I  
*Oxygen Uptake per Gm. of Fresh Tissue per Hour*

| Tissue                  | No added substrate | 4 mg. succinic acid |
|-------------------------|--------------------|---------------------|
|                         | c. mm.             | c. mm.              |
| Rat kidney.....         | 463                | 5932                |
| " liver.....            | 552                | 3896                |
| " brain (cerebrum)..... | 561                | 2568                |
| Chick kidney.....       | 745                | 7105                |
| " liver.....            | 901                | 4065                |
| " brain (cerebrum)..... | 563                | 2012                |
| " " (optic lobes).....  | 333                | 2022                |

TABLE II  
*Cyanide Inhibition on Succinoxidase*

|                           | Oxygen uptake per gm. fresh tissue per hr. |          |          |           |
|---------------------------|--|----------|----------|-----------|
|                           | Control                                    | m/100 CN | m/300 CN | m/1000 CN |
|                           | c. mm.                                     | c. mm.   | c. mm.   | c. mm.    |
| KOH in center cup.....    | 7205                                       | 6405     |          |           |
| No KOH in center cup..... | 5405                                       | 407      | 966      | 3940      |

were able to corroborate his findings in a more striking manner by eliminating the KOH from the center cups entirely. Oxygen uptake experiments without the CO<sub>2</sub> absorbers showed that there was little CO<sub>2</sub> evolution, in accordance with the fact that CO<sub>2</sub> is not evolved when succinic acid is oxidized to fumaric acid. It was thus possible to measure the effect of cyanide with no KOH in the CO<sub>2</sub> absorbers. The results of these experiments are shown in Table II.

Thus m/100 cyanide produced only 11 per cent inhibition when



KOH was in the center cup, while the same amount of cyanide produced an inhibition of 92 per cent in the absence of KOH.

In experiments with yeast (11) we showed that sodium selenite was toxic for systems oxidizing alcohol and acetic acid, although very slight toxicity was observed for the oxidation of lactic and

TABLE III

*Oxygen Uptake of Chick Kidney (40 Mg.) in 1 Cc. of M/30 Na and K Phosphate at pH 7.4*

4 mg. of succinic acid were used in each flask except those without substrate.

| Inhibitor            | Oxygen uptake per gm. fresh tissue per hr. |         |                            |       |        |
|----------------------|--|---------|----------------------------|-------|--------|
|                      | No substrate                               | Control | Concentration of inhibitor |       |        |
|                      |  |         | M/100                      | M/300 | M/1000 |
|                      | c.mm.                                      | c.mm.   | c.mm.                      | c.mm. | c.mm.  |
| Sodium selenite..... | 755  | 7012    | 415                        | 983   | 1820   |
| " selenate .....     | 917  | 7800    | 7080                       | 7323  | 8225   |
| " arsenite .....     | 583  | 6950    | 2120                       | 3300  | 4710   |
| " arsenate.....      | 779  | 7110    | 5560                       | 6463  | 7075   |
| " fluoride*.....     | 855  | 6920    | 6190                       | 6935  |        |

\* With 0.04 M fluoride the uptake was 4760 but this may have been partly due to a slight clumping of tissue which occurred.

TABLE IV

*Oxygen Uptake of Chick Kidney (140 Mg.) per Cc. with No Added Substrate*

| Inhibitor            | Oxygen uptake per gm. fresh tissue per hr. |       |       |        |        |
|----------------------|--|-------|-------|--------|--------|
|                      | Control                                    | M/100 | M/300 | M/1000 | M/3000 |
|                      | c.mm.                                      | c mm  | c.mm. | c.mm.  | c.mm.  |
| Sodium selenite..... | 2400                                       | 108   | 153   | 389    | 920    |
| " selenate.....      | 2570                                       | 2220  | 2230  | 2420   | 2540   |
| " arsenite.....      | 2310                                       | 317   | 610   | 1020   | 1990   |
| " arsenate.....      | 2580                                       | 2040  | 2300  | 2410   | 2590   |

pyruvic acids. It was suggested that selenite inhibited the glycolytic enzyme system as well as certain oxidizing systems. Experiments on succinate oxidation by yeast were not conclusive because yeast oxidizes succinate very slowly. A number of experiments have now been carried out to show the effect of selenium and arsenic on succinoxidase from animal tissues, with the homo-

genized tissue in low concentration. Selenium was used in the form of sodium selenite and sodium selenate and arsenic was used as sodium arsenite and sodium arsenate. Labes and Krebs (12) have shown that sodium selenite and tellurite are toxic to succinoxidase from muscle powder. These workers showed that the inhibitors acted on the dehydrogenase rather than on the cytochrome and indophenol oxidase. The results of our experiments are averaged in Table III. The averages of two experiments with sodium fluoride are also included.

Table III shows that with respect to succinic acid dehydrogenase, both elements are more toxic in the lower valence. In comparison with selenite, the selenate is almost non-toxic for the period included in the study. It has been known for some time that trivalent arsenic is much more toxic than pentavalent arsenic for Protozoa, yeasts, and bacteria, and it has been suggested that pentavalent arsenic is reduced to the trivalent form in the organism before exerting its toxic effects (Sollman (13)). The results in Table III indicate that the observations in regard to the effect of valence on toxicity may hold in the case of succinic acid dehydrogenase as well. However, it is not certain that the relation is specific for succinoxidase, because it is also found in the case of tissues with no added substrate (Table IV). There remains the possibility that the oxidations pass through the succinate-fumarate system (Annau *et al.* (4)).

It has also been shown that the selenate and arsenate were much less toxic than the corresponding "ite" salts in the case of yeast respiration on a glucose substrate (Potter (14)). It was found that sodium selenate exerted no appreciable toxic effect during the first 20 minutes, but by the end of 2 hours the oxygen uptake was only 60 per cent as great as that of the controls. With sodium selenite the inhibition began at once, so that at the end of 20 minutes the uptake was only 50 per cent as great as that of the controls. It thus seems quite likely that the selenate must be reduced to selenite before any toxicity results. In the case of the arsenic salts, it was found that the arsenate produced almost no inhibition even by the end of 2 hours, while the arsenite produced marked inhibition by the end of the first 20 minutes. Apparently the yeast organism is able to reduce the selenate more readily than the arsenate. The pharmacological differences which have

been observed between trivalent and pentavalent arsenic (13) are apparently closely bound up with the effect of these forms of the element on cellular oxidations. It is to be expected that the differences which are observed in oxygen uptake experiments or tests with Protozoa with the various forms of arsenic or selenium would be more apparent than those observed in feeding trials or injections of the salts. Thus Franke and Potter (15) did not differentiate between the toxicity of orally administered sodium selenite and selenate. A very careful comparison of the two compounds might actually indicate a greater toxicity for selenate than for selenite, since the latter might be reduced to the non-toxic elemental selenium by the intestinal flora more rapidly than the selenate.

TABLE V

*Effects of Dicarboxylic Acids on Succinic Acid Oxidation by Suspensions of Chick Kidney (40 Mg. per Cc.)*

Oxygen uptake per gm. of fresh tissue per hour.

| Succinic acid, $\mu$ .....     | None | 0.034<br>None | None | 0.034<br>0.034 | 0.034<br>0.0085 | 0.034<br>0.0021 |
|--------------------------------|------|---------------|------|----------------|-----------------|-----------------|
| Dicarboxylic acid, $\mu$ ..... |      |               |      |                |                 |                 |
| Oxalic acid.....               | 1036 | 7130          | 563  | 3566           | 5166            | 6343            |
| Malonic ".....                 | 1265 | 6990          | 453  | 1082           | 3210            | 5683            |
| Glutaric ".....                | 820  | 7273          | 589  | 5407           | 6370            | 7200            |
| Adipic acid.....               | 1325 | 6786          | 1739 | 5300           | 6406            | 6960            |
| L-Aspartic acid.....           | 936  | 7043          | 1653 | 6293           | 6620            | 6837            |
| L-Malic acid.....              | 1163 | 6992          | 3069 | 5808           | 6403            | 7020            |
| Fumaric ".....                 | 1085 | 7225          | 3550 | 5475           | 7010            | 7450            |

Our attention was next turned to the inhibition of succinic acid oxidation by malonic acid and other dicarboxylic acids. Quastel and Woolridge (4) studied the ability of a large number of compounds to inhibit the reduction of methylene blue by *Bacillus coli* on a succinic acid substrate. We have studied the oxidation of succinic acid in the presence of homologous dicarboxylic acids from oxalic acid up to and including adipic acid. The effect of aspartic acid ( $\alpha$ -aminosuccinic acid) was also included, as well as two acids which represent stages in the oxidation of succinic acid, namely fumaric and malic acids. A dilute suspension of chick kidney was used as the source of succinoxidase and the ability of the preparation to oxidize the various dicarboxylic acids alone was also measured. The results are shown in Table V.

The inhibition of succinic acid oxidation by malonic acid would seem to be easily explained on the basis of an affinity between the succinic acid dehydrogenase and malonic acid, owing to the similarity in configuration. Presumably the dissociation of the enzyme-substrate complex takes place when succinic acid has been dehydrogenated to fumaric acid, but since malonic acid cannot be dehydrogenated, it does not readily dissociate from the enzyme, and consequently prevents it from reacting with succinic acid. It was suggested by Quastel and Woolridge (5) that the enzyme affinity depended upon a  $-\text{C}-\text{CH}_2-\text{COOH}$  linkage, associated possibly with another carboxyl group. The results in Table V show the importance of both  $-\text{COOH}$  groups and the possibility that they may need to be fairly close to each other. Both oxalic and malonic acids seem to have a very high affinity for the enzyme and it may be pointed out that there is no  $-\text{CH}_2-$  group in oxalic acid. The inhibitory effects of oxalic acid are probably not due to the removal of calcium ions, since the buffer does not contain calcium and furthermore it was shown that calcium itself inhibits succinic acid oxidation to the extent of as much as 27 per cent at a concentration of  $\text{m}/600$  under conditions as in Table III. In the present studies, osmotic effects have been largely eliminated, owing to the rupture of most of the cells.

Glutaric acid, in which the two carboxyls are separated by three  $-\text{CH}_2-$  groups, might be expected to be oxidized to some extent by the enzyme, since there are adjacent  $-\text{CH}_2-$  groups and the carboxyls are separated by only one more  $-\text{CH}_2-$  than in succinic acid. Table V shows that glutaric acid is completely unoxidized by the enzyme, although there seems to be some affinity, since succinic acid oxidation is somewhat inhibited in the presence of the 5-carbon acid. Adipic acid, with four  $-\text{CH}_2-$  groups, appears to inhibit succinic acid oxidation to about the same extent as glutaric acid, but when it is the sole substrate it is slightly oxidized, while glutaric acid actually inhibited the oxygen uptake of the suspension with no added substrate. Aspartic acid resembled adipic acid in its action. The oxygen uptake of a system utilizing fumaric acid should theoretically be equal to or less than the uptake when malic acid is used, since the conversion of fumaric acid to malic acid involves only the addition of the elements of water. Actually we found a slightly greater uptake on fumaric acid than

on malic acid. In a paper by Green and Brosteaux (16) the same relation may be observed in the case of a reconstructed lactic acid dehydrogenase system tested with fumaric and malic acids. The dilute suspension of chick kidney probably retains some fumarase as well as some malic acid dehydrogenase activity, yet it is difficult to explain the inhibition of succinic acid oxidation if two distinct enzymes are involved. However, the occurrence of specific enzymes for fumaric and malic acids does not prevent the possibility of their adsorption on the succinic acid dehydrogenase, and their configuration makes this adsorption seem likely. The results in Table V illustrate the high specificity of the succinic acid dehydrogenase in so far as oxidation is concerned, but clearly indicate that the enzyme has a high affinity for dicarboxylic acids of 4 carbon atoms or less, and a lesser affinity for the 5- and 6-carbon acids.

The inhibition of the "no substrate" oxygen uptake by oxalic, malonic, and glutaric acids raises the question as to whether or not the compounds are specifically inhibiting succinic acid dehydrogenase, just as this question was raised in the case of selenium and arsenic inhibition. It was found that when the tissue concentration was increased in order to reduce the dilution effect and give an appreciable oxygen uptake in the absence of added substrate (as in Table IV), the malonic acid produced an inhibition of about the same magnitude as in Table V. It seems more likely that malonic acid should inhibit succinic acid dehydrogenase specifically than that it should inhibit respiration in general. The inhibition observed in the absence of added succinic acid may be the indirect result of the inability of the tissue to oxidize the succinic acid already present in the tissue. Green and Brosteaux (16) have shown that the oxidation of lactic acid is inhibited in a few minutes if pyruvic acid is allowed to accumulate.

#### SUMMARY

1. Various tissues from rats and chickens have been assayed for succinic acid dehydrogenase by the authors' method and the enzyme has been found to occur in greatest amounts in kidney, followed in order by liver and brain.

2. The enzyme system as it occurs in suspensions of homogenized chick kidney was found to be inhibited by cyanide, sodium

selenite, and sodium arsenite, while sodium fluoride, sodium selenate, and sodium arsenate were relatively non-toxic.

3. Malonic and oxalic acids were found to inhibit the oxidation of succinic acid by the system, while glutaric, adipic, aspartic, malic, and fumaric acids caused much less inhibition. Of the acids mentioned only malic and fumaric were oxidized by the preparation to a significant extent.

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## THE OPTICAL INVERSION OF *d*-HISTIDINE IN THE ANIMAL BODY\*

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Several years ago Berg and Potgieter suggested that such unnatural optical antipodes of essential amino acids as promote growth when fed as supplements in diets deficient in their natural isomers probably do so because they are made available by more or less complete conversion into the natural modification in the animal body (2). No actual proof of this assumption has thus far been established. The isolation of the acetyl derivative of *d*-phenylaminobutyric acid from the urine after the administration of pure *l*-phenylaminobutyric acid (17) constitutes the nearest approach, but the conditions are hardly analogous inasmuch as neither phenylaminobutyric acid isomer is of use to the body. Growth stimulation by an antipode does not preclude the possibility that the antipode might be used as such for tissue anabolism; nor does it oppose the less plausible conception that the unnatural amino acid might perform some function (or functions) ordinarily assumed by the natural modification, and thus conserve and render adequate for tissue synthesis the unavoidable traces of the latter in the diet (3).

The present paper describes studies which we believe show that *d*-histidine does undergo transformation into its natural *l* isomer in the growing rat.

### EXPERIMENTAL

The general plan of experimental procedure was as follows: A number of young rats, about 1 month of age, were placed on a

\* The experimental data are taken from a dissertation submitted by Ralph M. Conrad in June, 1936, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa.



diet containing only known traces of histidine. After 8 days, approximately half of the animals were killed and analyzed for histidine. The remaining rats were divided into three groups. One group was continued on the histidine-deficient diet; a second was fed a supplement of *l*-histidine; and the third, one of *d*-histidine. During the experimental period weight changes and food consumption were recorded. At the conclusion of the experiment, all of the animals were killed and analyzed for histidine. The net gain of histidine in the animals fed the *d* modification was estimated by subtracting the histidine present in the tissues at the outset of the experimental period and the traces of *l*-histidine in the total quantity of food consumed during the study from the histidine found in the rat at the conclusion of the experiment. Finally, the specific rotation of the histidine found in each rat grown on *d*-histidine was established.

The validity of the experimental data obviously depends upon (1) the optical purity of the *d*-histidine fed, (2) the analytical procedures for estimating the quantity of histidine in the rats and in the dietary components, and (3) the method used to establish the optical purity of the histidine isolated.

*Preparation and Properties of d- and l-Histidine Monohydrochloride*—The *l*-histidine monohydrochloride was prepared from commercial dried blood by the method of Cox, King, and Berg (4). The *d*-histidine was obtained as follows: *l*-Histidine was racemized by heating a solution containing 5 gm. of it per 100 cc. of 1:1 hydrochloric acid for 72 hours at 150°. The racemic histidine was isolated as the monohydrochloride (4). To a solution of 0.02 mole of the latter were added 0.01 mole of silver *d*-tartrate and 0.01 mole of *d*-tartaric acid. The mixture was shaken thoroughly, the silver chloride was filtered off, and the resulting histidine tartrate was fractionated as described by Pyman (13). The *d*-histidine hydrogen tartrate obtained was recrystallized from water four times to insure the complete removal of the *l*-histidine modification. The tartaric acid was removed by adding an excess of lead acetate. Excess lead was precipitated from the lead tartrate filtrate with hydrogen sulfide. The dihydrochloride was prepared from the lead sulfide filtrate by evaporating to dryness (under reduced pressure) several times with

an excess of hydrochloric acid, and was converted to the monohydrochloride by the aniline method (4).

The optical rotation of the *d*-histidine monohydrochloride, determined on (a) a 5 per cent solution in water containing 3 equivalents of HCl, was  $[\alpha]_D^{20} = -8.29^\circ$ , and on (b) an 8 per cent solution in water containing 1 equivalent of HCl,  $[\alpha]_D^{20} = -8.28^\circ$ . Under conditions similar to (a), Cox and Berg (3) found  $-8.14^\circ$ , Ehrlich (6)  $-7.89^\circ$ ; under conditions obtaining in (b), Cox and Berg found  $-8.25^\circ$ , and, for the corresponding levo compound, Abderhalden and Einbeck (1) found  $+8.28^\circ$ . The specific rotation of the *l*-histidine monohydrochloride prepared and used in these studies was  $[\alpha]_D^{20} = +8.28^\circ$  under the conditions described in (a).

*Preparation and Composition of Histidine-Deficient Diet*—The histidine-deficient casein hydrolysate which served as the chief dietary source of nitrogen was prepared by the following procedure. 1 kilo of casein was refluxed for 24 hours with 4 liters of water and 1 liter of concentrated sulfuric acid. After cooling, 1800 gm. of mercuric sulfate were added and the sulfuric acid concentration was reduced to about 5 per cent by adding 24 liters of water slowly, stirred vigorously the while to insure complete solution of the sulfate. The solution was placed in the refrigerator for 5 or 6 days to complete the precipitation of the histidine, the last of which separated very slowly. The mercuric sulfate precipitate was filtered off from the cold solution, the excess mercury was removed from the filtrate by shaking with hydrogen sulfide, the sulfate ion was exactly removed from the mercuric sulfide filtrate with barium hydroxide, and the barium sulfate filtrate was concentrated *in vacuo*. The residue was dried and ground. A 10 per cent solution of the product thus prepared gave a barely perceptible test by Hunter's method (9). So little histidine was present that its precipitation and subsequent analysis seemed less advisable than fixing its maximum limit by the Hunter (9) test. Such estimation indicated that not over 0.075 per cent could have been present in the hydrolysate. The latter was incorporated in a basal diet containing starch 39.5, sucrose 15.0, Crisco 19.0, cod liver oil 5.0, salt mixture (Hawk and Oser (8)) 4.5, agar 2.0, histidine-deficient casein hydrolysate 14.5, trypto-

phane 0.2, and cystine 0.3 per cent. The complete basal diet thus contained less than 0.011 per cent of histidine. Vitamin B complex was supplied by the separate daily feeding of two tablets, each composed of 50 mg. of yeast vitamin (Harris) and 50 mg. of starch. The histidine content of each tablet (determined by hydrolyzing 10 gm. lots of the vitamin concentrate and analyzing according to the procedure outlined below for estimating the histidine content of the rats) was 0.29 mg. and constituted the largest source of histidine in the basal diet.

Since, so far as we are aware, mercuric sulfate has not previously been used in removing histidine from protein hydrolysates intended for use in diets, a trial feeding test was made to establish the fact that other dietary requisites had not been removed in sufficient quantity to interfere with growth when the hydrolysate was supplemented with histidine and incorporated in the mixture indicated.

*Procedure for Histidine Determination*—The bodies of the rats (and the vitamin concentrate) were analyzed for histidine by hydrolyzing, precipitating with silver nitrate as directed by Vickery and Block (16), and determining the histidine in the precipitate by the Kapeller-Adler (10) Pulfrich photometer method, modified as described below.

In the estimations on the rats, the possibility of including histidine which was undergoing absorption was eliminated by removing the gastrointestinal tract with its contents from each animal immediately after it was killed for analysis. This procedure introduces a negative error in the absolute histidine analyses which is quantitatively greater in the larger animals killed at the termination of the studies than in the younger animals killed at the beginning. It should therefore add to, rather than detract from, the validity of positive inversion data. The larger rats (weighing usually about 100 gm. after removal of the alimentary tract) were ground and hydrolyzed separately by refluxing for 30 hours with 300 cc. of 8 N sulfuric acid. In analyzing the very small animals, several carcasses<sup>1</sup> were hydrolyzed and analyzed together. An aliquot of the hydrolysate was taken for Kjeldahl nitrogen determination and the remainder diluted to 4 liters, neutralized to

<sup>1</sup> The term carcass as used in this paper refers to the whole rat, minus only the gastrointestinal tract.

Congo red with barium hydroxide, and filtered. The barium sulfate filter cake was washed thoroughly and the filtrate and washings were concentrated *in vacuo* to 400 cc. Attempts were made to apply the Kapeller-Adler method directly to aliquots of this crude hydrolysate, but the presence of material other than histidine which absorbed large amounts of bromine, as well as the dark color of the hydrolysate itself, interfered enough to render the analytical results uncertain. These difficulties were avoided by first partially purifying and concentrating the histidine through the silver salt. The histidine was precipitated from the solution essentially according to the Vickery and Block (16) technique. 50 gm. of silver nitrate were dissolved in the concentrate and warm saturated barium hydroxide solution was added slowly with stirring until the solution became slightly alkaline to phenolphthalein. After standing in the refrigerator overnight, the silver salt was filtered off. The filtrate from the latter was routinely tested for histidine by Hunter's method (9), but none was ever found. Certainly only minute traces of histidine could have escaped detection. According to Vickery and Block the precipitation is complete.

The precipitate was suspended in 500 cc. of water, acidified to Congo red with sulfuric acid, and decomposed by shaking with hydrogen sulfide. After filtering out and washing the silver sulfide thoroughly, the filtrate and washings were decolorized with animal charcoal, condensed *in vacuo* to less than 100 cc., and made up to volume in a 100 cc. volumetric flask. 1 cc. of this solution was diluted to 10 cc. and used for histidine determination by the Kapeller-Adler procedure (10), modified in one detail. Preliminary work with the method as described in the literature indicated, as indeed Kapeller-Adler and others have pointed out, that enough bromine must be used to insure complete bromination of the histidine, but that any large excess of bromine must be avoided to obtain the maximum development of color during the subsequent treatment. In the original method, Kapeller-Adler used the color of excess bromine itself to indicate when the optimum amount had been added. This procedure was found too empirical and inexact to give consistently good results. The difficulty was obviated by adding a small but definite excess of bromine and, at the end of 10 minutes, destroying the unabsorbed

bromine by the addition of a drop of a saturated solution of arsenious oxide in a 10 per cent solution of ammonia. This insures complete bromination and at the same time prevents variations in excess of bromine which might otherwise influence color development when the ammonia-ammonium carbonate mixture is added and the solution is heated.

After some practise in applying the complete procedure as outlined, it was possible to obtain results having maximum variations of 5 per cent. The method was tested thoroughly on known solutions of histidine and on histidine additions to hydrolysates. In all cases the analyses were within the limits of error indicated. When the complete procedure was applied also to a sample of acid-precipitated casein containing 14.06 per cent of nitrogen, 2.16 per cent of histidine was found; thus, 4.16 per cent of the total N of the casein tested was in the form of histidine. Van Slyke (15) recommends the Osborne, Leavenworth, and Brautlecht (12) modification of the Kossel and Patten (11) method for determining histidine as preferable to his own nitrogen partition procedure. Using the former, he found 4.16 and 4.51 per cent or an average of 4.34 per cent of total casein N in the form of histidine N. With this our analysis agrees well.

So far as we are aware no data are available for suitable comparison with the histidine analyses which we obtained on rat carcasses. Our data show an average of approximately 0.46 per cent for young animals weighing from 30 to 40 gm.; of the total N found, 3.49 per cent was in the form of histidine. For the carcasses of rats weighing 101 to 144 gm., these values averaged somewhat lower, 0.41 per cent and 3.28 per cent, respectively.

*Procedure for Determining Optical Purity of Histidine*—The optical character of the histidine found in the rat tissues was established by converting it to the methyl ester dihydrochloride and polarizing. This compound was chosen because it can be prepared easily in uncontaminated form and because it has a higher specific rotation than either histidine monohydrochloride or histidine dihydrochloride.

The solution which was not needed for the colorimetric determination of the histidine was further purified by electrical transport, essentially as outlined by Cox, King, and Berg (4). The cell used in this case was smaller, each compartment containing

slightly over 100 cc. It was made of glass. The electrodes were of platinum. The cathode liquor was replaced by water every 6 hours; usually the center compartment was found free of histidine after 18 hours. The combined cathode liquors were concentrated *in vacuo* to 100 cc., and the histidine was precipitated by adding 30 cc. of 25 per cent mercuric sulfate in 4 N sulfuric acid. After 2 days in the refrigerator, the precipitate was filtered off, dissolved in 100 cc. of 1:4 hydrochloric acid, and the mercury precipitated by saturating with hydrogen sulfide. The mercuric sulfide was filtered off and any sulfate ion present was removed by adding a slight excess of barium chloride. The solution was decolorized with animal charcoal, filtered, and evaporated to dryness *in vacuo*. The histidine dihydrochloride was extracted and the barium chloride residue was washed with a total of 20 cc. of alcohol. The histidine was precipitated as the monohydrochloride by adding 1 cc. of aniline (4). After 2 days in the refrigerator, the monohydrochloride was filtered off, dried in the oven at 100°, taken up in 10 cc. of anhydrous methanol, and esterified by passing in dry hydrogen chloride gas for 3 hours while the solution was being gently refluxed (7). The solution was cooled, seeded with 1 mg. or less of the powdered compound, and placed in the refrigerator. Even with such seeding, the crystallization was sometimes very slow in starting. The solution was left in the refrigerator for at least a day, usually several, after crystallization had definitely begun. Finally the ester was filtered off, sucked dry, and washed with 3 cc. of a mixture of 1 part of methanol and 3 parts of ether. The crystallized product was dried in the oven at 100° and weighed. The yield was usually between 200 and 300 mg.

With such incomplete isolation, the question at once arose as to whether an optical isomer present in small quantity might not escape detection. However, when 25 mg. of pure *d*-histidine monohydrochloride were mixed with 475 mg. of the pure *l* compound and the mixture was esterified, the isolated product showed a markedly lower specific rotation (+8.7°) than did that similarly prepared from pure *l*-histidine (+9.66°). Furthermore, when 18 mg. of *d*-histidine were added to each of three ground rat carcasses weighing 150 to 200 gm. and the mixtures were subsequently carried through the hydrolysis and the isolation pro-

cedures described, the specific rotations of the methyl ester dihydrochlorides obtained were also distinctly lower ( $+8.7^\circ$ ,  $+8.2^\circ$ , and  $+8.3^\circ$ ). When *d*-histidine was present, precipitation (obviously of *dl*-histidine methyl ester dihydrochloride) occurred during the esterification procedure. Such early precipitation was not otherwise encountered.

*Growth Studies*—The twenty-seven young rats which were placed on the histidine-deficient diet were of the same age. Their individual weights ranged between 35 and 45 gm. They were kept in separate cages. The diet was fed *ad libitum*. Two vitamin tablets were supplied daily, as previously indicated. Distilled water was always available. The rats were weighed and food consumption was determined every 4 days. After 8 days on the histidine-deficient regimen, thirteen animals were killed for analysis and fourteen were retained for the growth studies. Of the latter, four animals were fed a diet in which 0.37 per cent of *d*-histidine, as the monohydrochloride, replaced an equal amount of the hydrolysate; three rats were started on a diet containing half of this amount of *d*-histidine, but were given the 0.37 per cent supplement after 28 days in order to stimulate better growth; four were placed on a similar ration containing 0.37 per cent of *l*-histidine; and three were continued on the basal diet. After 92 to 100 days on these diets, the animals were killed for analysis.

The growth and food consumption data are summarized in Table I. The animals on the deficient diet lost weight consistently and all but one eventually died. This constitutes biological evidence that the unsupplemented diet was definitely deficient in histidine. The diet of the animals in Series II of Cox and Berg (3) probably contained more histidine than that used here, since their deficient animals, after the first 8 days, gained weight slowly. Their use of whole yeast as a vitamin B source may account for the difference. The growth and food consumption data reported in this study agree in general with those obtained by Cox and Berg.

*Histidine Analyses*—Details of the analytical procedure have been described above. All of the animals fed histidine were fasted for 24 hours prior to analysis to reduce the content of absorbed, but unmetabolized histidine. For the younger animals which had been fed the histidine-deficient diet this seemed un-

necessary. As an additional safeguard, the gastrointestinal tract was stripped as free as possible of adhering tissue and removed and discarded. This procedure was applied also to the younger animals. To judge from Donaldson's data (5) and our own observations on the weights of several emptied gastrointestinal tracts of the 30 to 40 gm. and 100 to 145 gm. rats, the difference in mass during the 92 to 100 days growing period could not have

TABLE I  
*Growth and Food Consumption*

| Rat No.<br>and sex | Initial*<br>weight | Final<br>weight | Total food<br>consump-<br>tion | Histidine supplement                |
|--------------------|--------------------|-----------------|--------------------------------|-------------------------------------|
|                    | gm.                | gm.             | gm.                            | per cent                            |
| 307 ♀              | 30                 | 114             | 417                            | 0.37 d-, 92 days                    |
| 316 ♂              | 38                 | 118             | 403                            | Same                                |
| 320 ♀              | 32                 | 106             | 363                            | "                                   |
| 308 ♂              | 38                 | 102             | 304                            | 0.185 d-, 28 days; 0.37 d-, 64 days |
| 309 ♀              | 34                 | 144             | 447                            | 0.185 " 28 " 0.37 " 72 "            |
| 315 ♀              | 35                 | 118             | 434                            | Same                                |
| 314 ♀              | 32                 | 112             | 381                            | 0.37 d-, 100 days                   |
| 310 ♀              | 34                 | 101             | 357                            | 0.37 L-, 100 "                      |
| 311 ♂              | 37                 | 114             | 419                            | Same                                |
| 317 ♂              | 38                 | 144             | 367                            | "                                   |
| 324 ♂              | 38                 | 133             | 470                            | "                                   |
| 319 ♀              | 40                 | 38              | 170                            | None, 100 days                      |
| 318 ♀              | 37                 | 36              | 32                             | " died after 14 days                |
| 323 ♀              | 34                 | 30              | 85                             | " " " 42 "                          |

\* All animals were placed on the histidine-deficient diet for 8 days before initiating the experiment. During this period the average individual weight loss was 5.7 gm.

exceeded 4 per cent of the final live weight in any of the rats used by us. The purpose of combining four or five carcasses of the 30 to 40 gm. animals for analysis was to provide a sample of size approximating the anticipated average mass of the carcasses of the animals to be fed the diets containing histidine.

The nitrogen and histidine data are presented in Table II. In most instances there was a fair correlation between carcass weight and total nitrogen and histidine content. The somewhat



higher average nitrogen content of the young rats killed for analysis may be ascribed to their loss in weight, probably largely in the form of glycogen and fat, during their 8 day subsistence on the

TABLE II  
*Nitrogen and Histidine Contents of Rat Carcasses\**

|                                | Rat No. and sex                   | Carcass weight | Total N |                         | Histidine |                         | Histidine nitrogen |                  |
|--------------------------------|-----------------------------------|----------------|---------|-------------------------|-----------|-------------------------|--------------------|------------------|
|                                |                                   |                | gm.     | per cent carcass weight | mg.       | per cent carcass weight | mg.                | per cent total N |
| Controls, sacrificed at outset | 1 ♀, 2 ♂,<br>3 ♀, 4 ♀             | 102            | 3.84    | 3.77                    | 503       | 0.49                    | 136                | 3.54             |
|                                | 5 ♂, 6 ♂,<br>7 ♂, 8 ♂             | 101            | 3.52    | 3.49                    | 474       | 0.47                    | 128                | 3.64             |
|                                | 9 ♂, 10 ♂,<br>11 ♀, 12 ♂,<br>13 ♂ | 140            | 4.73    | 3.38                    | 567       | 0.41                    | 154                | 3.28             |
|                                | Average.....                      |                |         | 3.55                    |           | 0.46                    |                    | 3.49             |
| <i>d</i> -Histidine            | 307 ♀                             | 101            |         |                         | 440       | 0.44                    |                    |                  |
|                                | 316 ♂                             | 102            | 3.30    | 3.24                    | 377       | 0.37                    | 102                | 3.05             |
|                                | 320 ♀                             | 95             | 3.10    | 3.26                    | 407*      | 0.43                    | 110                | 3.24             |
|                                | 308 ♂                             | 87             |         |                         | 392       | 0.45                    |                    |                  |
|                                | 309 ♀                             | 128            | 3.89    | 3.04                    | 474       | 0.37                    | 128                | 3.29             |
|                                | 315 ♀                             | 106            | 3.41    | 3.34                    | 359       | 0.34                    | 97                 | 2.85             |
|                                | 314 ♀                             | 94             | 3.14    | 3.21                    | 415       | 0.44                    | 112                | 3.58             |
|                                | Average.....                      |                |         | 3.22                    |           | 0.41                    |                    | 3.20             |
| <i>l</i> -Histidine            | 310 ♀                             | 83             | 2.82    | 3.40                    | 370       | 0.45                    | 100                | 3.55             |
|                                | 311 ♂                             | 97             | 3.13    | 3.23                    | 415       | 0.43                    | 112                | 3.59             |
|                                | 317 ♂                             | 121            | 4.03    | 3.33                    | 489       | 0.40                    | 133                | 3.29             |
|                                | 324 ♂                             | 115            | 3.71    | 3.23                    | 429       | 0.37                    | 116                | 3.13             |
|                                | Average.....                      |                |         | 3.30                    |           | 0.41                    |                    | 3.39             |
| No histidine                   | 319 ♀                             | 31             | 1.01    | 3.26                    | 106       | 0.36                    | 29                 | 2.84             |

\* The gastrointestinal tract with its contents was removed before the carcass was weighed. It was not included in the analyses.

histidine-deficient diet. The nitrogen content of the older animals is lower and nearer that usually found in well nourished rats, but still slightly higher on the average. For this the 24 hour fast may

have been partly responsible. Most of the animals were not fat, but some variation was noted. No essential differences were apparent between the rats fed *l*-histidine and those fed the *d* modification, either in nitrogen or in histidine content. Comparable data on the gross histidine content of normal rats are apparently not available. In comparison with data on muscle tissue of the rat and with data on other tissues of other species, the results seem reasonable. The average histidine content found was roughly about one-third of the gross arginine content of rats reported by Scull and Rose (14).

TABLE III  
*Histidine Balances on Rats Fed d-Histidine*

| Rat No. and sex | Maximum<br><i>l</i> -histidine fed* | Histidine content of rats |       | Histidine arising<br>from <i>d</i> -histidine<br>source |
|-----------------|-------------------------------------|---------------------------|-------|---|
|                 |                                     | Initial†                  | Final |   |
|                 | mg.                                 | mg.                       | mg.   | mg.   |
| 307 ♀           | 99                                  | 105                       | 440   | 236   |
| 316 ♂           | 98                                  | 133                       | 377   | 146   |
| 320 ♀           | 94                                  | 112                       | 407   | 201   |
| 308 ♂           | 87                                  | 133                       | 392   | 172   |
| 309 ♀           | 107                                 | 119                       | 474   | 248   |
| 315 ♀           | 106                                 | 123                       | 359   | 190   |
| 314 ♀           | 100                                 | 112                       | 415   | 208   |

\* Each gm. of basal diet contained not over 0.11 mg. of *l*-histidine; 0.58 mg. of histidine was added daily in the two vitamin pills fed.

† The total live weight of the thirteen controls was 441 gm.; their total carcass content of histidine was 1544 mg. The average content of histidine per gm. of live weight was thus 3.5 mg. Initial histidine in the animals fed *d*-histidine was therefore calculated as gm. of initial live weight times 3.5.

Table III includes the estimate of histidine originally present in the rats fed *d*-histidine, the maximum *l*-histidine calculated to have been fed them, the histidine actually found in their carcasses, and finally (by subtracting from the last the sum of the original histidine present plus the *l*-histidine fed), the histidine which must have come from the *d*-histidine fed. From 58 to 111 per cent more histidine was present than could be accounted for by the sum of the histidine originally present plus that fed as *l*-histidine. We believe that this difference is significant and cannot

be set aside by undetected errors or inadequacy of methods employed.

*Optical Nature of Histidine Isolated*—Table IV presents the specific rotations of the methyl ester dihydrochlorides prepared from the various rats fed *d*-histidine, from those fed *l*-histidine, and from pure *l*-histidine itself. Comparisons of these several values indicate clearly that no appreciable amount of *d*-histidine could have been present in the tissues of the rats fed *d*-histidine. As we have previously indicated, the method is capable of detecting even small traces of added *d*-histidine.

TABLE IV  
*Optical Rotation of Histidine Methyl Ester Dihydrochlorides*

| Prepared from rats fed |                                 |                     |                                 | Synthesised from<br><i>l</i> -histidine<br>[α] <sub>D</sub> <sup>20°</sup> |
|------------------------|---------------------------------|---------------------|---------------------------------|--|
| <i>d</i> -Histidine    |                                 | <i>l</i> -Histidine |                                 |  |
| Rat No. and sex        | [α] <sub>D</sub> <sup>20°</sup> | Rat No. and sex     | [α] <sub>D</sub> <sup>20°</sup> |  |
|                        | <i>degrees</i>                  |                     | <i>degrees</i>                  | <i>degrees</i>   |
| 307 ♀                  | +9.15                           | 310 ♀               | +9.70                           | +9.75  |
| 316 ♂                  | +9.60                           | 311 ♂               | +9.50                           | +9.45  |
| 320 ♀                  | +9.60                           | 317 ♂               | +9.40                           | +9.65  |
| 308 ♂                  | +9.50                           | 324 ♂               | Sample lost                     | +9.80  |
| 309 ♀                  | +9.50                           |                     |                                 |  |
| 315 ♀                  | +9.50                           |                     |                                 |  |
| 314 ♀                  | +9.70                           |                     |                                 |  |
| Average ....           | +9.51                           |                     | +9.53                           | +9.66  |

\* Concentration 1 gm. per 100 cc. of solution; water used as solvent.

The data presented account definitely for an inversion of 9 to 16 per cent of the *d*-histidine fed. These calculated values must be regarded as minimal, since any loss of *l*-histidine formed by inversion, but subsequently eliminated or transformed metabolically, would result in a low apparent inversion. It is obvious from comparisons of the growth rates of our *d*-histidine animals with those of Cox and Berg on rats fed various levels of *l*-histidine (3) that the apparent *d*-histidine inversion calculated to have occurred in our studies could not alone have been responsible for the growth rates observed. Either some utilization of uninverted *d*-histidine for metabolic purposes apart from tissue synthesis must

have occurred, or a considerably greater synthesis of *l*-histidine than that demonstrated must have taken place.

#### SUMMARY

Comparisons have been made between the histidine increments in the tissues of rats fed histidine-deficient diets supplemented with *d*-histidine and the cumulative traces of *l*-histidine fed in such diets. The basal diets alone were too low in histidine to support growth; when either *d*- or *l*-histidine was added, growth ensued. From 58 to 111 per cent (or 88 per cent, on the average) more histidine was laid down in the *d*-histidine animals than could be attributed to *l*-histidine of dietary origin. Optical measurements showed that the histidine present in the tissues of these animals was essentially pure *l*-histidine. Hence *l*-histidine must have been formed metabolically for tissue synthesis from the *d*-histidine fed.

The findings support the view that antipodes which can replace their natural essential amino acid isomers are themselves converted, at least partially, into the needed natural enantiomorph.

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## A PHOTOELECTRIC MICROCOLORIMETER

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The photoelectric colorimeter recently described by one of us (1) required a sample of at least 6 cc. and could not, therefore, be used in a number of important techniques in which only small amounts of solution are available. In this paper we shall describe an attachment which allows this colorimeter to be used with (a) small quantities (0.1 to 2.0 cc.) of faintly colored solutions, and (b) thin layers (0.1 to 1.0 mm. thick) of deeply colored media such as whole blood. With this attachment, the principles of photoelectric filter photometry may be applied to all the systems which can be analyzed by the spectrophotometer, or by visual photometers such as the Pulfrich. The techniques for which the microcolorimeter is most valuable fall into three groups: (a) those involving the use of small amounts of solutions whose properties would be radically altered by further dilution, *e.g.* the microcolorimetric determination of pH and the determination of blood volume by the dye method; (b) microchemical techniques in which the only means of decreasing the amount of material required is to reduce the volume of the final colored solution to a minimum, *e.g.* the microcolorimetric determination of hemoglobin and the determination of vitamin A in blood by the antimony trichloride method; and (c) colorimetric analysis of deeply colored media, *e.g.* the determination of reduced hemoglobin, carbon monoxide hemoglobin, and methemoglobin in whole blood.

### *Description of Apparatus<sup>1</sup>*

The median vertical section of Fig. 1 shows that the illuminating system, glass color filters, and filter-changing mechanism

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<sup>1</sup> Drawings and constructional details are available to those who wish to construct this new attachment.

are the same as in the previous instrument. The long axis of the body is, however, horizontal instead of vertical, so that the direction of the light beam is parallel to the axis of the absorption cells instead of at right angles to it as in the original design. The bakelite test-tube holder has been replaced by a rectangular compartment containing a sliding metal carriage in which two flat bottomed tubular absorption cells are mounted. Each cell fits into a removable hard rubber bushing, the lower end of which

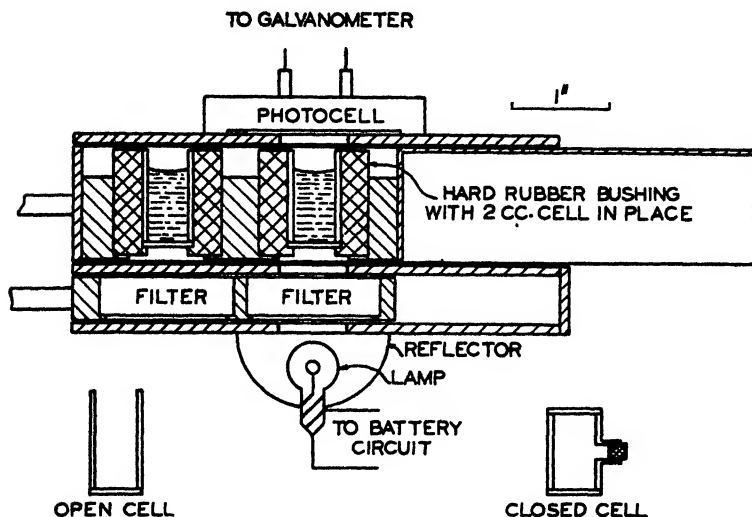


FIG. 1. Median vertical section through the body of the microcolorimeter. A pair of 2 cc. cells is shown in their hard rubber bushings. For the open cell the inside height is 25.5 mm.; the outside height, 27.0 mm. For the closed cell the inside height is 20.0 mm.; the outside height, 23.0 mm. The inside diameters of the cells are as follows: 11.28 mm. for the 2.0 cc. cell; 8.00 mm. for the 1.00 cc. cell; and 5.05 mm. for the 0.4 cc. cell.

acts as a diaphragm to limit the cross-section of the light beam to a circle whose diameter is equal to that of the inside of the cell. The carriage is moved back and forth by means of a rack and pinion, while a ball-and-spring click mechanism automatically centers first one cell then the other in the path of the beam of light.

When the hard rubber bushings are removed, the upper portion of the carriage forms a shallow compartment in which may be

placed flat cells for use with thin layers of opaque liquids. In addition, cells of almost any shape may be used if special bushings or cell carriages are made to accommodate them.

*Absorption Cells*—The tubular cells are made by fusing or cementing plane-parallel glass bottoms onto pieces of Jena KPG Precision Bore glass tubing. The dimensions of three useful cells are shown in Fig. 1. The inside diameters are accurate to 0.01 mm. and the cells are filled to a height of 20.0 mm. by 2.0, 1.0, and 0.4 cc. respectively. The height of the absorbing layer is kept constant by filling the cells with accurate pipettes. The upper edge of each cell is painted black to prevent the passage of light upward through the glass wall of the tube. We have found these open ended cells both accurate and convenient, but whenever the accurate measurement of the volume of the sample is difficult, one may use similar cells closed by flat plates at *both* ends and equipped with a side arm for filling. Special bushings may easily be made for such cells, and it may also be more convenient to operate the instrument in a vertical position when using them.

The thin cells which we have used are made from microscope slides, ground to enclose a cavity about 15 mm. in diameter. We have not yet found it necessary to use liquid layers less than 0.5 mm. thick, but the carriage compartment has purposely been made large enough to hold a cell of the type recently described by Drabkin and Austin (2), since we believe that this is the best cell yet designed for direct colorimetric measurements on very thin layers of whole blood.

*Electrical Circuit*—The microattachment has been designed for mounting on the same panel as the standard instrument, so that the same switches, rheostats, battery, and galvanometer may be used for both. In order to allow the use of the very small cells, even with the most selective filters, it may be advisable to use a slightly more sensitive galvanometer than the one originally recommended. The Rubicon type 3403-H is most satisfactory.

*Method of Operation*—The only important change in the operation of the instrument is due to the necessity for pipetting accurately the amount of sample used. Accurate fine tipped pipettes must be used to fill both the blank cell (which contains



solvent only) and the sample cell. The galvanometer is adjusted to 100 with the blank cell in position; the sample cell is then racked into place and the new galvanometer reading used in conjunction with a previously prepared calibration chart in the usual manner. To insert and remove the cells the carriage is racked out to its fullest extent, but is never completely removed from the instrument. As a general rule one should use the largest cell which will meet the requirements of a particular problem, since this not only decreases the error in pipetting the sample, but also decreases the voltage at which the lamp must be operated.

#### DISCUSSION

The data already presented (1) on the accuracy and precision of the original instrument are equally applicable to the performance of the microattachment, since numerous parallel determinations have proved that there is no significant difference between the results obtained with the two instruments. It may be recalled that the main limiting factor in the precision of the original model was the unavoidable variation in the absorption test-tubes. This source of error has been eliminated in the micromodel by the use of precision bore tubing, but a new source of error of about the same size has been introduced by the necessity for pipetting small volumes of the sample fluid. We have found that a precision of better than 0.5 per cent can be consistently maintained by using fine tipped, calibrated pipettes with the 2 cc. cells.

An important advantage of the open ended absorption cell, in addition to its simplicity and convenience, is the fact that it can be used satisfactorily with much smaller amounts of fluid than its maximum capacity, since the height of the absorbing column can be calculated as long as the volume of the sample is known. For example, it is possible to use as little as 0.1 cc. in a 1.0 cc. cell, provided special care is taken in measuring this very small volume. When partly filled cells are used, it is essential that an exactly equal volume of solvent be placed in the blank cell, so that the two optical paths are identical. The multiplication factor which must be used in such cases cannot be calculated accurately by linear proportionality, on account of reflection effects which occur at the curved surface of the meniscus. It is, however, a

simple matter to determine the correct factor by a control experiment on the same type of solution.

Even when the open cells are filled to the maximum height of 20 mm., accurate results can only be obtained if the shape of the meniscus is the same in the samples as in the blank. Since surface tension is an important factor in determining the shape of the meniscus, it is essential that the surface tension be kept constant by careful control of the concentrations of all the constituents of the samples and the blank. In any case, the formation of a symmetrical meniscus depends on careful delivery of the solution and the use of scrupulously clean glassware. In any determination in which unavoidable variations in surface tension or difficulties in the pipetting of the samples might result in errors in the use of open cells, one should use the closed cells in spite of the slightly greater difficulty involved in filling and cleaning them.

#### SUMMARY

1. An attachment is described which allows the photoelectric colorimeter to be used with samples of 0.1 to 2.0 cc., and also with very thin layers of deeply colored material such as whole blood.

2. In the new attachment the same color filters and electrical accessories are used as with the instrument previously described, but the design of the absorption cell holder has been altered to allow the use of a wide variety of microcells.

3. The accuracy of the original instrument has not been impaired by the use of smaller volumes, although the field of application of the principles of photoelectric filter photometry has now been extended to include all the colorimetric measurements for which the spectrophotometer or any other type of photometer may be used.

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# **SYNTHESIS OF 1-*d*-RIBOSIDOURACIL. INTERACTION OF ACETOBROMO-*d*-RIBOSE AND 2,4-DIETHOXYPYRIMIDINE\***

BY GUIDO E. HILBERT AND CARL E. RIST

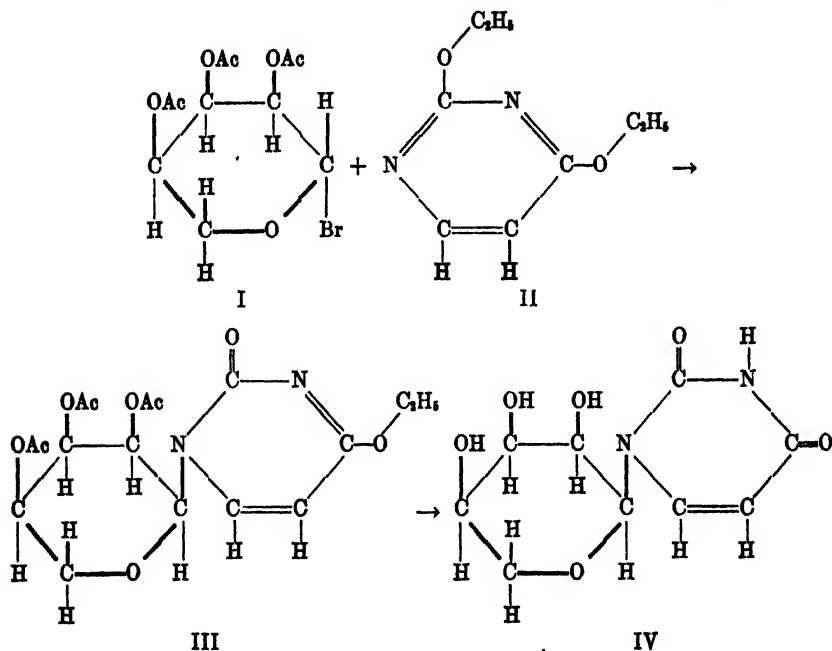
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Prior to the time this work was started, the only investigation which might be considered to have offered any information on the ring structure of the ribose portion of the nucleosides was that of Levene and Sobotka (1). They reported 1,3-dimethylxanthosine, derived from guanosine, to be identical with the theophylline-*d*-riboside, which was prepared by deacetylating the product obtained in the interaction of the silver salt of theophylline with acetobromo-*d*-ribose. Since a synthetic nucleoside prepared in this manner would, according to modern concepts of the ring structures of sugars, be expected to be a pyranoside, it follows, because of the supposed identity of the above products, that the ribose group in guanosine should be similarly constituted. Assuming that the ribose in uridine has a like structure, a promising procedure for the synthesis of this nucleoside seemed to be one analogous to that used for the synthesis of 1-*d*-glucosidouracil (2) and the interaction of acetobromo-*d*-ribose and 2,4-diethoxypyrimidine was therefore studied. In the meantime, however, convincing evidence was obtained by Levene and Tipson (3) and by Brederick (4) that the sugar in uridine, as well as in the other nucleosides, is in the furanoside form. Hence a synthetic 1-*d*-ribosidouracil prepared from acetobromo-*d*-ribose would probably be an isomer of uridine, instead of being identical with it. Our study confirms this.

\* The numbering of the pyrimidine cycle is in conformity with the scheme used by *Chemical Abstracts*, but is different from that used by many workers in the field.

The interaction of acetobromo-*d*-ribose (I) and 2,4-diethoxypyrimidine (II) proved to be quite complex. A small quantity of uracil, which was apparently formed by some obscure hydrolytic process, separated from the reaction syrup. Subsequent to its removal, an ethoxytriacetylribosidopyrimidine crystallized. This, because of its ease of hydrolysis, was evidently structurally different from the naturally occurring nucleosides (for further discussion see below). No additional crystalline material could be obtained from the residual syrup, even though it was subjected



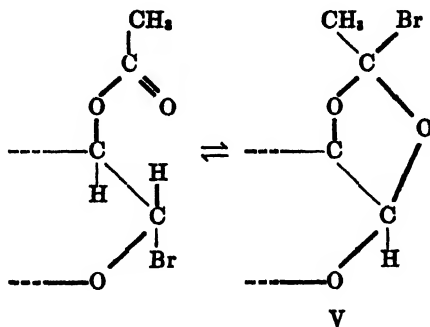
to various physical treatments and allowed to sit in the cold for a few years. The product was finally hydrolyzed with alcoholic hydrochloric acid in order to convert 1,2-dihydro-2-keto-4-ethoxy-1-triacetyl-*d*-ribosidopyrimidine (III) into 1-*d*-ribosidouracil (IV) and to degrade contaminating side-products. After removal of the solvent, the crude product was worked up by using a procedure essentially similar to that of Levene and La Forge (5) for isolating uridine from a yeast nucleic acid hydrolysate, except that our material was taken through the acetyl instead of the

benzoyl stage. In one of the steps of this process 1-ethyluracil was isolated, thus affording evidence for the probable presence of 1,2-dihydro-2-keto-1-ethyl-4-ethoxypyrimidine as a by-product in the above reaction. Concentration of the neutral filtrate obtained after decomposition of the basic lead acetate precipitate with sulfuric acid gave a syrup from which crystalline 1-*d*-ribosidouracil (IV) was readily obtained. The synthesis of this product may be expressed as shown in Formulas I to IV.

The chemical properties of 1-*d*-ribosidouracil are similar to those of uridine. Neither of these compounds reduces Fehling's solution or responds to the Wheeler-Johnson color test, and both are (a) extremely stable towards strong acid, (b) precipitated by basic lead acetate, and (c) readily acetylated. 1-*d*-Ribosidouracil gives a crystalline triacetyl derivative, m. p. 184–185°,  $[\alpha]_D^{20} = -25.1^\circ$  (CHCl<sub>3</sub>), whereas triacetyluridine as obtained by Levene and Tipson (3) is an amorphous compound (no polarimetric data reported). The physical properties of 1-*d*-ribosidouracil and uridine are quite different. 1-*d*-Ribosidouracil melts at 257° and uridine at 165°. Moreover the former is much less soluble in water and in alcohol than the latter, and they separate from solvents in decidedly different crystalline forms. The most remarkable difference was encountered in the determination of the specific rotation; that of the synthetic product was found to be  $-141.0^\circ$  in water, whereas the value reported for uridine (6) in the same solvent is  $+4.0^\circ$ . Whether this indicates that the uracil moiety in these compounds has opposite configurations about carbon atom (1) of ribose remains to be determined.

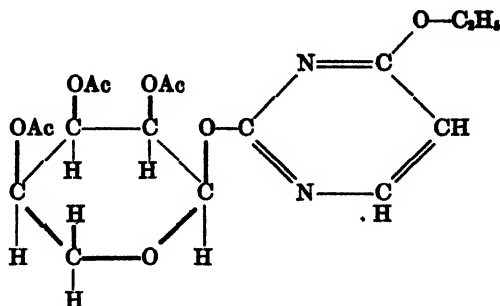
Although the interaction of several acetobromoglycosides with 2,4-diethoxypyrimidine has been studied, only acetobromo-*d*-ribose yielded, in addition to the normal product (III), a crystalline, anomalous, labile glycosidopyrimidine. This ethoxytriacetyl-*d*-ribosidopyrimidine on treatment with alcoholic hydrochloric acid in the cold was hydrolyzed to give uracil and presumably ethyl-*d*-riboside. With 0.05 *N* alkali the ribose-pyrimidine linkage was ruptured, and the pyrimidine isolated and identified as 1,2-dihydro-2-keto-4-ethoxypyrimidine (7). This result shows that the ribose must originally have occupied either position (1) or (2) of the pyrimidine cycle. As the acetobromo derivatives of a number of sugars, as ribose, mannose, etc., under certain

conditions seem to exhibit the following type of tautomeric equilibrium (8, 9) the formation of a product, in which the bromine atom in (V) is replaced by the pyrimidine ring, might well be



expected in the reaction. Such an "orthoacetate" structure was seriously considered, but the evidence obtained would seem to eliminate this possibility. Orthoacetates in the sugar series, as for example  $\gamma$ -triacetylmethyl-*d*-ribose, are characterized by the stability towards alkali of one of the "acetyl" groups, specifically that utilized in ring formation. In contrast to this behavior all of the acetyl groups of the labile nucleoside are readily removed even when alkali as dilute as 0.025 *N* was used in the hydrolysis. Additional evidence against the orthoacetate formulation was obtained by ultraviolet absorption spectra studies. From the fundamental work of Austin (10) on the relation between absorption and substitution in the pyrimidine ring, the orthoacetate would be expected to give practically the same absorption curve as 1,2-dihydro-2-keto-1-methyl-4-alkoxypyrimidine. Professor Emma P. Carr of Mt. Holyoke College very kindly made available to us absorption curves of the labile nucleoside and for purposes of comparison of 1,2-dihydro-2-keto-4-ethoxy-1-triacetyl-*d*-xylo- and 1,2-dihydro-2-keto-4-ethoxy-1-tetraacetyl-*d*-glucosido-pyrimidine. The absorptions of the last two compounds are practically identical both in position and in shape with that of 1,2-dihydro-2-keto-1-methyl-4-methoxypyrimidine, whereas the peak of absorption of the labile nucleoside lies about 150-160 $\text{\AA}$ . closer to the visible region of the spectrum and practically overlaps the absorption of 2,4-diethoxypyrimidine. Because of this

similarity we are inclined to believe that the product is 4-ethoxy-2-triacetyl-*d*-ribosidopyrimidine (VI). In support of this view, as (VI) may be considered to be the ammono analogue of a sugar



VI

acetate, is the ease with which the ribose is removed from the pyrimidine ring by both dilute acid and dilute alkali.

The problem dealing with the synthesis of the naturally occurring pyrimidine nucleosides may be divided into two parts: (a) the discovery of a method for attaching the carbohydrate to position (1) of an appropriately substituted pyrimidine and (b) a means for preparing a suitable ribose derivative in the furanoside form. Results obtained in this work and in the general study of the interaction of acetobromo sugars with 2,4-diethoxypyrimidine (11) show quite definitely that a method is now available for dealing successfully with the first part of the above problem. The solution of the second part requires considerable experimentation.

We wish to express our appreciation to Dr. R. T. Milner and Mrs. M. S. Sherman for determining the microanalyses recorded.

#### EXPERIMENTAL

*d*-Ribose was prepared from guanosine according to the method of Levene and Clark (12). Pure guanosine was easily obtained from yeast nucleic acid by following their directions. In our hands, however, difficulty was encountered in the isolation of adenosine. The picrate of this when initially precipitated was contaminated with another more insoluble picrate, which could not be removed by ordinary crystallization. *d*-Ribose was acetyl-



ated in the same manner as described by Levene and Tipson (9). After the reaction mixture was poured on ice, it was found to be more convenient to separate the crystalline tetraacetyl derivative by filtration rather than by extraction with chloroform. Tetraacetyl-*d*-ribose was converted to acetobromo-*d*-ribose, which crystallized as large thick rectangular plates when the toluene-hydrobromic acid-acetic acid solution was being concentrated under diminished pressure.

*Interaction of Acetobromo-d-Ribose and 2,4-Diethoxypyrimidine*—To 4 cc. of 2,4-diethoxypyrimidine were added 3.47 gm. of finely divided acetobromo-*d*-ribose and the mixture heated at 65° for 18 hours; the mixture was frequently stirred until the acetobromo-*d*-ribose, which dissolves very slowly, was in solution. During the course of the reaction a strong odor of ethyl bromide was noticeable. The final reaction mixture was a brown syrup containing a finely divided colorless solid. After treatment with an equal volume of dry ether, the solid (0.05 gm.) was removed by filtration, washed well with ether and alcohol, and recrystallized from 2 cc. of water (decolorized with bone-black). It did not melt at 300°, gave a Wheeler-Johnson color test, and was identified as uracil.

*Analysis*— $C_4H_4O_2N_2$ . Calculated. C 42.84, H 3.60, N 25.00  
Found. " 43.09, " 3.50, " 24.99

*4-Ethoxy-2-Triacetyl-d-Ribosidopyrimidine*—The filtered syrup after standing for a short time deposited a colorless crystalline solid. This was filtered (yield 0.76 gm.) and recrystallized from 25 cc. of 95 per cent ethyl alcohol from which it separated as long flat plates, m. p. 162.5°. The ribosidopyrimidine is only slightly soluble in water and ether but very soluble in chloroform. It is levorotatory, showing an  $[\alpha]_D^{20}$  value of  $-66.2^\circ$  (1.0102 gm. in 10 cc. of chloroform solution in a 2 dm. tube gave a reading of  $13.37^\circ$ , to the left). Further recrystallization did not alter the specific rotation nor raise the melting point. An alcoholic solution of it reduces Fehling's solution. After treatment of the riboside with 5 per cent hydrochloric acid and subsequent concentration of the solution to dryness, the residual hydrolytic products respond to the Wheeler-Johnson color test. That uracil

is actually formed by this treatment was shown in another experiment, where it was isolated and identified.

*Analysis*—

$C_{17}H_{23}O_5N_3$ . Calculated. C 51.23, H 5.57, N 7.04,  $OC_2H_3$  11.31  
Found. " 51.63, " 5.32, " 7.18, " 11.30

*Hydrolysis*—In order to serve as a control, the acid and alkaline hydrolysis of 1,2-dihydro-2-keto-4-ethoxy-1-triacetyl-*d*-xylosido-pyrimidine was studied simultaneously with that of 4-ethoxy-2-triacetyl-*d*-ribosidopyrimidine. When a solution of 10 cc. of water, 0.5 cc. of 0.018 *N* sulfuric acid, and 0.075 gm. of the ribosido derivative was heated in a sealed tube at 100° for 2 hours, the acetic acid liberated was 6.8 per cent of that required for three acetyl groups; under identical conditions the xylosido derivative gave 3.9 per cent. When the ribosido derivative was heated with 0.040 *N* sulfuric acid for 4 hours, 89.5 per cent of the acetic acid required by theory for complete hydrolysis was liberated. The acetyl groups of the xylosido and ribosido derivatives were completely hydrolyzed when a 0.075 gm. specimen dissolved in 20 cc. of acetone was allowed to stand with 30 cc. of 0.042 *N* sodium hydroxide for 4 hours in an ice bath. The details of one hydrolysis experiment in which the pyrimidine fragment was isolated are as follows: a solution of 0.150 gm. of the riboside in 20 cc. of acetone (purified) and 20 cc. of 0.102 *N* sodium hydroxide was set in an ice bath for 4 hours. The reaction mixture was then neutralized with 9.14 cc. of 0.105 *N* sulfuric acid, with methyl orange as the indicator. The amount of acetic acid generated was 95.3 per cent of that required by theory, assuming that 3 molecules were liberated from 1 molecule of the riboside.

*Isolation of 1,2-Dihydro-2-Keto-4-Ethoxypyrimidine*—After the above neutral solution was made slightly alkaline, it was placed in the ice chest for 2 days, then neutralized, and concentrated under diminished pressure. The residue was thoroughly triturated with 25 cc. of chloroform and the extract evaporated. A crystalline deposit remained and this was dissolved in about 1 cc. of hot water. On cooling, flat colorless plates separated; yield 20 mg.; m. p. 167–168°; when mixed with an authentic specimen of 1,2-dihydro-2-keto-4-ethoxypyrimidine there was no depres-

sion in the melting point. The product gave with bromine water and a solution of barium hydroxide the characteristic color reaction previously described for this compound.

*Analysis*— $C_8H_8O_2N_2$ . Calculated. C 51.40, H 5.76, N 20.00  
Found. " 51.26, " 5.79, " 19.94

*Isolation of 1-d-Ribosidouracil*—After removal of the crystalline riboside, the syrup even on standing for a long time deposited no additional crystalline material. It was dissolved in 25 cc. of absolute ethyl alcohol and treated with dry hydrogen chloride for 30 minutes. The solution immediately turned black and became quite warm. As no crystalline matter separated, the alcohol was evaporated by heating on a steam bath. The black syrupy residue was dissolved in alcohol and the solution again concentrated. Acetylation of the residual gum was accomplished by treating the product with 25 cc. of anhydrous pyridine and 25 cc. of acetic anhydride; on the addition of pyridine some uracil separated and this was removed. After standing for 24 hours the major portion of the solvent was removed by distillation at diminished pressure and the residue poured into ice water with vigorous stirring. The aqueous mixture was extracted with chloroform; the extract washed with water, and finally concentrated *in vacuo*. In order to remove the last traces of chloroform, alcohol was added and removed by distillation. The dark brown gum was dissolved in about 200 cc. of 50 per cent alcohol, treated with 8 gm. of barium hydroxide hydrate, and the mixture heated under a reflux for 3 hours. The hot reaction mixture was filtered. To the filtrate were added additional barium hydroxide and then an aqueous solution containing 20 per cent lead acetate until maximum precipitation was attained; an excess of either barium hydroxide or lead acetate was avoided. The colorless, finely divided precipitate was collected and washed well with water. It was decomposed by suspending in dilute sulfuric acid and stirring. The excess sulfuric acid was neutralized with barium carbonate and the filtrate treated with 1 per cent sulfuric acid until no further precipitate was formed. Concentration of the filtered solution at diminished pressure gave a pale yellow syrup that readily crystallized. Treatment with alcohol and collection of the crystals was followed by two crystallizations from 50 per cent

aqueous methyl alcohol. Anhydrous, colorless, glistening plates were obtained that melted at 257–258°; the melt on cooling resolidified and when reheated melted at 256°. The solubility of 1-*d*-ribosidouracil in water (0.1 gm. in about 2 to 3 cc.) is considerably less than that of uridine and like uridine it does not respond to the Wheeler-Johnson color test. It is levorotatory, showing an  $[\alpha]_D^{20}$  value of  $-140.0^\circ$  ( $c = 2.20$  in distilled water).

*Analysis*— $C_8H_{11}O_4N_3$ . Calculated. C 44.24, H 4.95, N 11.48  
Found. " 44.31, " 5.07, " 11.51

*1-Triacetyl-d-Ribosidouracil*—1-*d*-Ribosidouracil was acetylated with acetic anhydride in pyridine. The solution after standing for 24 hours was poured into iced water. As no precipitate was formed, the aqueous solution was extracted with chloroform. The extract was washed with water and dried over sodium sulfate. Removal of the solvent left a syrup which when worked with alcohol and ether crystallized in sheaves of colorless needles. The acetyl derivative was recrystallized from a solution consisting of 1 part of alcohol and 4 parts of ether; when pure it was slightly soluble in cold alcohol and soluble in hot alcohol and cold chloroform. Solvent of crystallization was removed by heating *in vacuo* at 100°; loss 4.8 per cent. The product occurs in two isomorphous forms. When the material was heated very slowly, it melted sharply at 184–185°, resolidified on cooling, and melted again at 184–185°; however, on rapid heating it melted at about 140°, slowly solidified, and melted at 184–185°. The specific rotation is  $[\alpha]_D^{20} = -25.1^\circ$  ( $c = 1.69$  in U.S.P. chloroform).

*Analysis*— $C_{18}H_{19}N_3O_7$ . Calculated. C 48.63, H 4.90, N 7.56  
Found. " 48.91, " 5.04, " 7.53

The acetyl derivative when subjected to alcoholic hydrochloric acid was converted to 1-*d*-ribosidouracil, m. p. 257°.

#### SUMMARY

1-*d*-Ribosidouracil (probably a pyranoside) was isolated from the hydrolyzed interaction product of acetobromo-*d*-ribose and 2,4-diethoxypyrimidine. Although the chemical properties of this synthetic nucleoside are similar to those of uridine (1-*d*-ribosidouracil-furanose form), the physical properties are mark-

edly different. From this it is evident that some form of ribose other than acetobromo-*d*-ribose is required for the synthesis of uridine. From the products of the above reaction, there was isolated directly a riboside, which is rapidly hydrolyzed by both dilute alkali and dilute acid. The evidence obtained suggests that it is 4-ethoxy-2-triacetyl-*d*-ribosidopyrimidine. As a whole the results indicate that a method is now available for treating successfully with that phase of the problem of the synthesis of pyrimidine nucleosides involving the introduction of a sugar in position (1) of the pyrimidine cycle.

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## QUANTITATIVE INVESTIGATIONS OF AMINO ACIDS AND PEPTIDES

### III. APPARENT ACID DISSOCIATION CONSTANTS IN AQUEOUS FORMALDEHYDE SOLUTION\*

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Los Angeles)*

(Received for publication, September 9, 1936)

The apparent acid dissociation constants of a number of amino acids were reported in Paper II (1). From a comparison of these data with those<sup>1</sup> of other investigators it was found that the  $pK'_a$  values decreased with increasing concentrations of formaldehyde. Although both Harris (2) and Levy (3) have derived generalizations for the more exact correlation of these factors, it was considered desirable to extend the observations of these investigators and, if possible, to determine quantitatively the relation between apparent dissociation constants and formaldehyde concentrations.

#### EXPERIMENTAL

The purity of the amino acids was established by formol-glass electrode titrations, with the modified glass electrode apparatus and the experimental technique described in earlier papers (1, 4). In determining apparent acid dissociation constants, 0.5 equiva-

\* A preliminary report was given before the Protein Conference at Stanford University, June 6, 1936.

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The authors are indebted to Professor G. Ross Robertson for the loan of his glass electrode apparatus and to Mr. A. Loshakoff for assistance in some of the preliminary phases of the experiments.

<sup>1</sup> In a private communication from Professor M. Levy it was stated that the following additional  $pK'_a$  values had been obtained: alanine 6.91, valine 7.41. The concentration of the formaldehyde was 10 per cent in each case.

lent of standard base was added to the aqueous solution of the amino acid, the calculated volume of neutral, approximately 37 per cent formaldehyde transferred from a calibrated burette to the amino acid solution, the mixture diluted to 50 ml. with neutral distilled water, and the solution stirred continuously for intervals of 10 to 15 minutes until the voltage became constant. The apparent acid dissociation constants were determined from

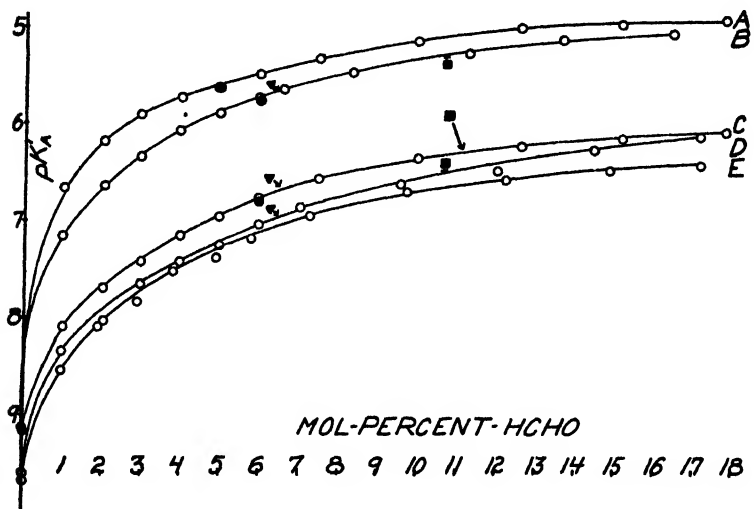


FIG. 1. Curves showing the relation between  $pK'_a$  and mole per cent formaldehyde. The data are plotted according to the following notations: Curve A, *dl*-serine; Curve B, glycine; Curve C, *dl*-phenylalanine; Curve D, *dl*-alanine; Curve E, *dl*- $\alpha$ -aminobutyric acid.  $\circ$  represents data of the present authors;  $\bullet$  those of Dunn and Loshakoff (1);  $\blacktriangle$  Levy (3, 5); and  $\blacksquare$  Harris (2). The values for  $pK'_a$  at 0 per cent formaldehyde (water) were taken from a previous paper (1).

the expression,  $pH = pK'_a$ , which was assumed to be valid under the conditions of the experiments. All determinations were made in an air-conditioned sub-basement room where the temperature was  $22^\circ \pm 0.5^\circ$ .

Previously, the purest commercial formaldehyde was treated with basic magnesium carbonate to remove formic acid. However, it was shown that the filtered solution was slightly alkaline ( $pH$  7.69), owing, presumably, to dissolved basic magnesium

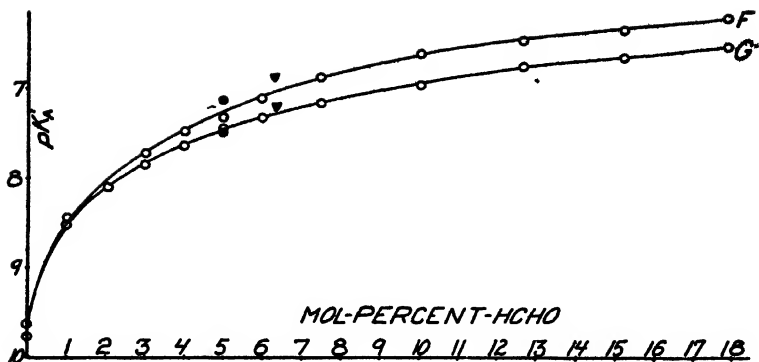


FIG. 2. Curves showing the relation between  $pK'$  and mole per cent formaldehyde. Curve F represents data for  $dl$ - and  $l$ -leucine and  $dl$ - and  $l$ -norleucine; Curve G,  $dl$ -valine and  $dl$ -norvaline.  $\circ$  represents data of the present authors;  $\bullet$  those of Dunn and Loshakoff (1) referring to  $dl$ -norleucine and  $dl$ -valine;  $\blacktriangle$  data of Levy (3, 5) referring to  $l$ -leucine and  $dl$ -valine. The values for  $pK'$  at 0 per cent formaldehyde (water) were taken from a previous paper (1).

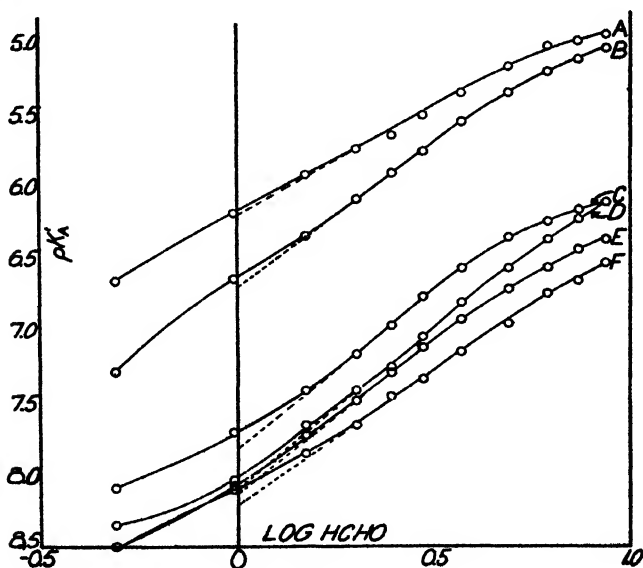


FIG. 3. The variation of  $pK'$  with the logarithm of the formaldehyde concentration. Formaldehyde is in moles per liter. Curve A represents data for  $dl$ -serine; Curve B, glycine; Curve C,  $dl$ -phenylalanine; Curve D,  $dl$ -alanine; Curve E,  $dl$ - $\alpha$ -aminobutyric acid; Curve F,  $dl$ -valine and  $dl$ -norvaline. The curve from which the constant for  $dl$ - and  $l$ -leucine and  $dl$ - and  $l$ -norleucine was determined has been omitted from the graph to avoid confusion.



carbonate. In the present experiments, neutral formaldehyde was prepared by contact with Merck's ignited reagent aluminum oxide. A glass electrode determination showed that the pH of the filtered solution was 6.85 after 15 minutes, 6.96 after 30 minutes, and 6.96 after 40 minutes. After longer intervals, the pH dropped slowly, indicating gradual oxidation of the formaldehyde by atmospheric oxygen.

TABLE I  
*Experimental Data from Analyses of Amino Acids*

| Amino acid                               | Per cent* of theoretical equivalent weight | Amino acid                | Per cent* of theoretical equivalent weight |
|--|--|---------------------------|--|
| Glycine†                                 | 99.80                                      | <i>l</i> -Norleucine**    |  |
| <i>dl</i> -Alanine                       | 99.85                                      | <i>dl</i> -Leucine§       | 99.99                                      |
| <i>dl</i> - $\alpha$ -Aminobutyric acid† | 100.11                                     | <i>l</i> -Leucine††       | 100.07                                     |
| <i>dl</i> -Valine§                       | 99.84                                      | <i>dl</i> -Serine¶        | 100.04                                     |
| <i>dl</i> -Norvaline                     | 99.93                                      | <i>dl</i> -Phenylalanine§ | 100.04                                     |
| <i>dl</i> -Norleucine¶                   | 99.72                                      |                           |  |

\* Equivalent weights were determined by the formol titration-glass electrode method of Dunn and Loshakoff (4).

† Prepared and analyzed by A. Loshakoff.

‡ Prepared by N. L. Smith.

§ Prepared by Amino Acid Manufacturers, University of California at Los Angeles.

|| Prepared by D. B. Tyler.

¶ Prepared by F. J. Ross and analyzed by A. Loshakoff.

\*\* Prepared by M. J. Naiditch.  $[\alpha]_D^{25} = -24.0^\circ$  (20 per cent HCl) (0.2551 gm. in 25 ml. of solution).

†† Prepared by W. H. Swanson and purified by repeated recrystallization from hydrochloric acid. Contained less than 0.003 per cent sulfur.

Summaries of the authors' experimental results are given in Figs. 1 to 3 and Tables I to V. The values listed in Table I are the average figures from duplicate determinations for which deviations ranging from 0.04 to 0.18 and averaging 0.11 per cent were found. The constants in Table II are the average figures from duplicate determinations for which deviations ranging from 0.00 to 0.02  $pK'_a$  units were observed. The average deviation between duplicate determinations was 0.004  $pK'_a$  unit. In all cases corrections were made for the percentage purity of amino acids.

## DISCUSSION

From an interpretation of the pH values in glycine solutions at varying formaldehyde concentrations, Harris concluded that a

TABLE II  
*Apparent Acid Dissociation Constants of Amino Acids in Aqueous Formaldehyde*

| Mole per cent HCHO* | Glycine | Al-<br>anine | dl- $\alpha$ -<br>Amino<br>butyric<br>acid | dl-<br>Valine | dl-<br>Nor-<br>valine | dl-<br>Nor-<br>leu-<br>cine | l-Nor-<br>leu-<br>cine | dl-<br>Leu-<br>cine | l-Leu-<br>cine | dl-<br>Serine | dl-<br>Phenyl-<br>alanine |
|---------------------|---------|--------------|--|---------------|-----------------------|-----------------------------|------------------------|---------------------|----------------|---------------|---------------------------|
| 0.99                | 7.16    | 8.36         | 8.52                                       | 8.52          | 8.51                  | 8.43                        | 8.42                   | 8.44                | 8.44           | 6.66          | 8.09                      |
| 2.04                | 6.64    | 8.04         | 8.06                                       | 8.10          | 8.10                  | 8.10                        | 8.10                   | 8.10                | 8.11           | 6.18          | 7.70                      |
| 2.96                | 6.34    | 7.65         | 7.72                                       | 7.84          | 7.85                  | 7.72                        | 7.71                   | 7.71                | 7.68           | 5.92          | 7.42                      |
| 3.95                | 6.08    | 7.42         | 7.48                                       | 7.65          | 7.65                  | 7.48                        | 7.48                   | 7.48                | 7.50           | 5.74          | 7.16                      |
| 4.95                | 5.90    | 7.26         | 7.28                                       | 7.45          | 7.45                  | 7.32                        | 7.31                   | 7.32                | 7.32           | 5.64          | 6.97                      |
| 5.94                | 5.74    | 7.04         | 7.12                                       | 7.32          | 7.34                  | 7.11                        | 7.10                   | 7.10                | 7.10           | 5.50          | 6.76                      |
| 7.46                | 5.54    | 6.80         | 6.92                                       | 7.15          | 7.15                  | 6.87                        | 6.87                   | 6.85                | 6.85           | 5.34          | 6.56                      |
| 10.0                | 5.34    | 6.56         | 6.71                                       | 6.94          | 6.95                  | 6.61                        | 6.61                   | 6.60                | 6.62           | 5.16          | 6.35                      |
| 12.7                | 5.20    | 6.36         | 6.56                                       | 6.74          | 6.74                  | 6.45                        | 6.45                   | 6.45                | 6.45           | 5.03          | 6.24                      |
| 15.2                | 5.11    | 6.22         | 6.44                                       | 6.64          | 6.64                  | 6.34                        |                        | 6.33                | 6.32           | 4.98          | 6.16                      |
| 17.9                | 5.04    | 6.10         | 6.36                                       | 6.52          | 6.51                  | 6.21                        |                        | 6.20                | 6.20           | 4.94          | 6.13                      |

\* Mole per cent formaldehyde is (moles of formaldehyde)/(moles of formaldehyde plus moles of water)  $\times 100$ .

TABLE III  
*Changes in Apparent Acid Dissociation Constants with Changes in Formaldehyde Concentration*

| Amino acid                      | Change in pK's<br>Range in mole per cent formaldehyde |         |         |          |          |         |
|---------------------------------|---|---------|---------|----------|----------|---------|
|                                 | 0.5-2.0   | 1.0-4.0 | 2.0-8.0 | 8.0-12.0 | 4.0-16.0 | Average |
| Glycine                         | 0.82  | 1.11    | 1.18    | 1.09     | 1.00     | 1.04    |
| dl-Alanine                      | 0.68  | 0.98    | 1.19    | 1.22     | 1.21     | 1.06    |
| dl- $\alpha$ -Aminobutyric acid | 0.81  | 1.16    | 1.10    | 1.08     | 1.04     | 1.05    |
| dl-Valine*                      | 0.56  | 0.82    | 1.03    | 1.06     | 1.05     | 0.90    |
| dl-Norleucine†                  | 0.76  | 1.03    | 1.18    | 1.23     | 1.18     | 1.08    |
| dl-Serine                       | 0.61  | 0.82    | 0.89    | 0.84     | 0.78     | 0.79    |
| dl-Phenylalanine                | 1.07  | 1.09    | 1.25    | 1.07     | 1.00     | 1.10    |
| Average.....                    |   |         |         |          |          | 1.02    |

\* The figures for dl-valine and dl-norvaline are identical.

† The figures for dl-norleucine, dl-leucine, l-leucine, and l-norleucine are identical.

4-fold increase of formaldehyde concentration results in 1 unit decrease of  $pK'_a$ . Since hydrogen ion concentration could not

TABLE IV  
Formol Titration Constants

Based on the relationship,  $pK'_a = -\log K_1K_2 - 2 \log (CH_2O)$ , at 22°.

| Amino acid                              | -Log $K_1K_2$ |         | Amino acid             | -Log $K_1K_2$ |         |
|---|---------------|---------|------------------------|---------------|---------|
|   | Levy          | Authors |                        | Levy          | Authors |
| <i>dl</i> -Alanine                      |               | 8.09    | Phenylaminoacetic acid | 6.90          |         |
| <i>dl</i> - $\alpha$ -Aminobutyric acid |               | 8.13    | <i>dl</i> -Serine      |               | 6.20    |
| Glycine                                 | 6.65          | 6.71    | <i>l</i> -Tyrosine     | 8.45          |         |
| <i>dl</i> -Leucine*                     | 7.87          | 7.99    | <i>dl</i> -Valine†     |               | 8.20    |
| <i>dl</i> -Phenylalanine                | 7.57          | 7.79    |                        |               |         |

\* The values for *dl*-leucine, *l*-leucine, *l*-norleucine, and *dl*-norleucine are identical.

† The values for *dl*-valine and *dl*-norvaline are identical.

TABLE V  
Coefficients of Apparent Acid Dissociation Constant Equations of Amino Acids\*

| Amino acid                              | <i>a</i> | <i>b</i> | <i>k</i> |
|---|----------|----------|----------|
| Glycine                                 | 4.491    | -3.330   | 26.52    |
| <i>dl</i> -Alanine                      | 5.232    | -5.650   | 50.52    |
| <i>dl</i> - $\alpha$ -Aminobutyric acid | 5.763    | -3.782   | 35.17    |
| <i>dl</i> -Valine                       | 5.717    | -6.043   | 53.89    |
| <i>dl</i> -Norvaline                    | 5.664    | -6.398   | 57.38    |
| <i>dl</i> -Norleucine                   | 5.399    | -5.042   | 45.90    |
| <i>l</i> -Norleucine                    | 5.090    | -6.483   | 58.09    |
| <i>dl</i> -Leucine                      | 5.430    | -4.761   | 43.57    |
| <i>l</i> -Leucine                       | 5.421    | -4.823   | 44.10    |
| <i>dl</i> -Serine                       | 4.437    | -3.832   | 27.29    |
| <i>dl</i> -Phenylalanine                | 5.658    | -2.394   | 22.57    |

\* Apparent dissociation constant equations:  $xy - ax - by - k = 0$ , where *x* is mole per cent formaldehyde, *y* is  $pK'_a$ , and *a*, *b*, and *k* are numerical constants.

be determined with high accuracy by the colorimetric method employed by this author, it was recognized that this generalization is only an approximation. Although  $pK'_a$  values at 16 per

cent formaldehyde were reported for alanine, phenylalanine, tyrosine, aspartic acid, and glutamic acid, the relation between formaldehyde concentration and amino acid constants was not studied in these cases.

A more precise expression of these relations

$$\text{pK}'_a = -\log K_1K_2 - 2 \log F$$

was developed by Levy from a consideration of the equilibrium reactions assumed to be set up in a system composed of amino acid zwitter ion, amino acid anion, formaldehyde, monoformaldehyde-amino acid anion, and diformaldehyde-amino acid anion. By plotting experimental  $\text{pK}'_a$  values against the logarithms of the formaldehyde concentrations, drawing smooth curves through the points, and extrapolating to  $\log F = 0$  those portions of the curves which reached or approached a slope of  $-2$ , the constants  $-\log K_1K_2$  were read at the intercepts. It was stated that the latter may be used to determine  $\text{pK}'_a$  values at a given formaldehyde concentration and that the equation to which they apply is valid for the amino acids studied from about 5 to 20 per cent (3 to 13 mole per cent) formaldehyde.

The average change of  $\text{pK}'_a$  with 4-fold changes in formaldehyde concentration was stated by Harris to be approximately 1 and by Levy (5) 1.2. Glycine alone was utilized by Harris, while presumably glycine, alanine, valine, leucine, tyrosine, phenylalanine, glutamic acid, and phenylamino-acetic acid were used by Levy for this purpose. The authors' results (Table III) with eleven different amino acid types indicate that the average  $\text{pK}'_a$  change is 1.02. However, it is evident that the changes in  $\text{pK}'_a$  vary widely with different amino acids and with different 4-fold changes in formaldehyde concentration. Hence, it is believed that little significance is attached to the figure representing the average change for one or a collection of amino acids.

As shown by the curves in Fig. 3 and the data in Table IV, the authors' experimental values are well defined by Levy's general expression relating  $\text{pK}'_a$  terms and formaldehyde concentrations. However, it may be noted that the  $-\log K_1K_2$  constants calculated from the present data are uniformly somewhat larger numerically than Levy's comparable values. A plausible explanation for these small differences may possibly be that the authors'

formaldehyde solutions were nearly neutral (pH 6.96), while those employed by Levy had a pH between 5 and 6. Furthermore, the present experiments were so designed that appreciable oxidation of the formaldehyde was avoided. In Levy's experiments, the formaldehyde was continuously in contact with the atmosphere during a series of consecutive analyses. Hence, it is not improbable that some formic acid may have been formed under these conditions.

It appears that the present results confirm Levy's statement that his equation is valid for the amino acids studied over a limited range of formaldehyde concentrations. However, beyond concentrations of 3 to 10 mole per cent formaldehyde, the experimental  $pK'_a$  values and those calculated from the equation become increasingly divergent. For this reason, it seemed desirable to provide expressions by which the  $pK'_a$ -formaldehyde relations would be accurately defined for a wider range of formaldehyde concentrations. Hence, the exact equations represented in Table V have been established and found to fit the experimental data obtained from measurements with the amino acids listed, with an average deviation of  $\pm 0.02$   $pK'_a$ .

It may be concluded from the data presented herewith that valine and norvaline, leucine and norleucine, and similar isomeric amino acids have identical apparent acid dissociation constants in formaldehyde. This result is not unexpected in view of the knowledge that, within the limits of experimental accuracy, the analogous normal and isobutyric, normal and isovaleric, and similar types of organic acids have the same acid dissociation constants in water. Also, it is of interest to note that identical constants were found for the optically active and inactive forms of certain amino acids.

While the purity of optically active amino acid samples cannot be determined unequivocally since absolute criteria for this purpose are not available, it is evident that significant differences in  $pK'_a$  values beyond the probable experimental error would have appeared if the material analyzed had contained appreciable quantities of the corresponding antipodes.

Harris' conclusion that the  $pK'_a$  of an amino acid solution to which alkali and formaldehyde have been added is independent of the ratio of the two latter components was acceptable to Levy in

all cases except for glycine. It was stated by Levy (3) that, "There is evidence in this case that the formaldehyde compound is polymerized to a marked extent and the values of the constants are dependent to some extent on the actual glycine concentration." However, Levy presented no experimental data in support of this conclusion. No indication of a glycine abnormality was found in the present experiments.

While it has been found in previous work (4) that the amphoteric amino acids may be accurately titrated with alkali at formaldehyde concentrations from approximately 9 to 10 per cent by weight (5.7 to 6.4 mole per cent), the bearing of the present studies on formol titrations cannot be determined completely at the present time. Investigations on this problem and on the titration of amino acid mixtures are planned for the immediate future.

#### SUMMARY

1. The generalization of Harris, describing the relation between the apparent acid dissociation constants of the amphoteric amino acids and formaldehyde concentrations, has been confirmed and extended.

2. A general equation relating  $pK'_a$  and formaldehyde concentration evolved by Levy from theoretical considerations has been shown to be valid for the amino acids studied between 3 and 10 mole per cent formaldehyde.

3. Exact equations have been formulated which described the behavior of certain amino acids in formaldehyde at concentrations ranging from about 1 to 18 mole per cent.

4. It has been shown that isomeric forms of the amphoteric amino acids have identical apparent acid dissociation constants at any given formaldehyde concentration between the imposed limits.

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## THE CONCENTRATION OF TOTAL CHOLESTEROL IN THE BLOOD SERUM\*

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It is agreed by most investigators that healthy persons may differ markedly in the concentration of total cholesterol in the blood serum, though none has recorded such large variations as were reported recently by Page, Kirk, Lewis, Thompson, and Van Slyke (1) (from 109 to 376 mg. per 100 cc.) and by the author (2) (from 132 to 392 mg. per 100 cc.). No non-lipid constituent of the serum varies so widely under physiological conditions. Most of the published data were obtained from single determinations on each of a number of subjects, and the question arises, therefore, whether the wide range in cholesterol concentration represents a constitutional difference among individuals or a variability in each individual from time to time. As far as the author is aware the only observation bearing on the point is that of Gardner and Gainsborough (3) who called attention to the constancy of the fasting values for total cholesterol concentration of the plasma in one of their subjects over a period of 3 years. In seven analyses the maximum deviation from the average was 15.0 per cent.

In the course of various studies of cholesterol metabolism under way in this laboratory from two to ten determinations of the concentration of total cholesterol in the serum, separated by intervals up to 28 months, were carried out in 25 young (19 to 43 years), healthy, adult persons of both sexes with the method of Schoenheimer and Sperry (4). The data (Table I) show that the variation is far less in a given individual than is that among differ-

\* This investigation was made possible by the support of the Josiah Macy, Jr., Foundation.



TABLE I  
*Total Cholesterol Concentration in Blood Serum*

| Subject No.* | Time after 1st analysis | Total cholesterol | Variation from average | Subject No. | Time after 1st analysis | Total cholesterol | Variation from average |
|--------------|-------------------------|-------------------|------------------------|-------------|-------------------------|-------------------|------------------------|
|              | mos.                    | mg. per 100 cc.   | per cent               |             | mos.                    | mg. per 100 cc.   | per cent               |
| 1-a†         | 0                       | 195.2             | -2.6                   | 61-a†       | 0                       | 336.0             | -0.6                   |
| 1-b†         | ½                       | 195.7             | -2.4                   | 61-b†       | 9                       | 319.0             | -5.6                   |
| 1-c†         | 1                       | 208.5             | +4.0                   | 61-c†       | 9½                      | 359.0             | +6.2                   |
| 1-d          | 7                       | 187.0             | -6.7                   | 62-a        | 0                       | 233.7             | +9.8                   |
| 1-e          | 9                       | 209.0             | +4.2                   | 62-b        | 9½                      | 192.0             | -9.8                   |
| 1-f          | 9½                      | 188.3             | -6.1                   | 64-a        | 0                       | 243.6             | -2.9                   |
| 1-g          | 10                      | 201.5             | +0.5                   | 64-b        | 28                      | 258.0             | +2.9                   |
| 1-h          | 10½                     | 216.7             | +8.1                   | 69-a†       | 0                       | 177.5             | +9.7                   |
| 1-i          | 18                      | 196.5             | -2.0                   | 69-b†       | ½                       | 146.0             | -9.7                   |
| 1-j          | 26                      | 206.5             | +3.0                   | 70-a†       | 0                       | 201.3             | +1.6                   |
| 2-a†         | 0                       | 334.3             | +2.0                   | 70-b†       | ½                       | 189.8             | -4.2                   |
| 2-b          | 6                       | 322.5             | -1.6                   | 70-c†       | 19                      | 203.5             | +2.7                   |
| 2-c          | 9                       | 329.0             | +0.4                   | 71-a†       | 0                       | 168.5             | -1.6                   |
| 2-d          | 9½                      | 253.0§            |                        | 71-b†       | ½                       | 168.5             | -1.6                   |
| 2-e          | 10                      | 321.5             | -1.9                   | 71-c†       | 19                      | 176.5             | +3.1                   |
| 2-f          | 26                      | 331.2             | +1.1                   | 72-a†       | 0                       | 179.5             | +1.3                   |
| 6-a          | 0                       | 131.5             | -7.5                   | 72-b†       | 19                      | 174.8             | -1.3                   |
| 6-b          | 10                      | 140.5             | -1.1                   | 73-a†       | 0                       | 171.0             | -6.1                   |
| 6-c          | 28                      | 154.3             | +8.6                   | 73-b†       | 18                      | 193.3             | +6.1                   |
| 7-a†         | 0                       | 269.0             | +8.2                   | 77-a        | 0                       | 202.3             | +0.7                   |
| 7-b          | 7                       | 264.0             | +6.2                   | 77-b        | 18                      | 199.4             | -0.7                   |
| 7-c          | 9                       | 247.0             | -0.6                   | 82-a        | 0                       | 249.5             | -2.9                   |
| 7-d          | 9½                      | 232.8             | -6.3                   | 82-b        | 17                      | 264.3             | +2.9                   |
| 7-e          | 10                      | 229.8             | -7.5                   | 83-a†       | 0                       | 197.8             | +0.1                   |
| 8-a          | 0                       | 207.0             | -2.5                   | 83-b†       | 17                      | 197.3             | -0.1                   |
| 8-b          | ½                       | 213.1             | +0.4                   | 84-a        | 0                       | 225.3             | -2.8                   |
| 8-c          | 10                      | 200.3             | -5.7                   | 84-b        | 15                      | 238.5             | +2.8                   |
| 8-d          | 25                      | 221.8             | +4.5                   | 85-a        | 0                       | 175.8             | -12.3                  |
| 8-e          | 27                      | 219.3             | +3.3                   | 85-b        | 15                      | 225.0             | +12.3                  |
| 9-a†         | 0                       | 249.5             | -2.3                   | 86-a†       | 0                       | 191.0             | -9.4                   |
| 9-b†         | ½                       | 250.0             | -2.1                   | 86-b†       | 14                      | 230.5             | +9.4                   |
| 9-c          | 9                       | 266.8             | +4.5                   | 87-a        | 0                       | 175.8             | +1.6                   |
| 50-a         | 0                       | 325.5             | +3.2                   | 87-b        | 14                      | 170.3             | -1.6                   |
| 50-b         | 8                       | 305.3             | -3.2                   | 88-a†       | 0                       | 195.8             | -3.3                   |
| 60-a         | 0                       | 186.8             | +6.7                   | 88-b†       | 13                      | 209.0             | +3.3                   |
| 60-b         | 6                       | 160.5             | -8.3                   | 89-a        | 0                       | 187.4             | +0.4                   |
| 60-c         | 12                      | 177.9             | +1.6                   | 89-b        | 11                      | 185.9             | -0.4                   |

\* The numbering is the same as was used in Table I of a previous publication (2). Subject 3, on whom several analyses were carried out, is omitted because he developed a spontaneous pneumothorax, of which the cause could not be established, shortly after the last determination.

† Samples were taken during fasting; the others at varying intervals after meals.

‡ Female subjects.

§ Omitted from the average; see foot-note 2

ent individuals. The maximum deviation in any subject (12.3 per cent from the average in Subject 85) is much less than the maximum deviation from the average of the entire series (62.2 per cent in Subject 61, Sample c). In an attempt to evaluate the difference statistically the standard deviation of the values for each subject<sup>1</sup> was calculated with the formula of Fisher (5) for small series of observations:  $\sigma = \sqrt{\sum d^2 / (n - 1)}$ . The average of the individual standard deviations was 12.1 mg. per 100 cc. in contrast with the standard deviation of the entire series (53.0 mg. per 100 cc.).

In interpreting the findings it must be borne in mind that apparent variations of appreciable magnitude may occur from hour to hour in a fasting person. The amount of cholesterol in the serum was determined at hourly intervals during the morning in three subjects without breakfast. The maximum deviation from the average for each subject was 3.3, 5.5, and 5.7 per cent, respectively. The variation is considerably greater than the probable error of analysis and must be ascribed in part at least to unknown factors, possibly to changes in the hydration of the serum. In a larger series even greater deviations may be anticipated, since there is no reason to suppose that the maximum was reached in this limited number of experiments. It is remarkable, therefore, that in seventeen<sup>2</sup> of the twenty-five subjects included in the present series the maximum deviation from the average was 6.2 per cent or less; *i.e.*, the variation over long intervals of time was no greater than might be anticipated in a single morning.

Somewhat greater deviations occurred in the other eight subjects, up to 12.3 per cent in Subject 85. The larger fluctuations may possibly have been associated with unrecognized pathological

<sup>1</sup> In many instances this involved the calculation of the standard deviation of only two observations. The validity of this approach was tested by arranging the values of the entire series in pairs at random and calculating the standard deviation of each pair. The average was 44.3 mg. per 100 cc., a value which is not greatly different from the standard deviation of the series as calculated in the usual manner (53.0 mg. per 100 cc.).

<sup>2</sup> This tabulation includes Subject 2 who showed the greatest deviation in the entire series in Sample d. In view of the striking constancy of the other values in this subject it seems likely that an error occurred in this particular determination and it appears justifiable to omit it from the average.

disturbances. All of the subjects were members of the faculty or technical staff or students of the College of Physicians and Surgeons. They were in good health as far as they knew but this was not confirmed by medical examination.

The rather large deviation in the female Subjects 69 and 86 may perhaps be related to the menstrual cycle (6), especially in Subject 69, in whom the two analyses were only about 2 weeks apart, but in the remaining seven female subjects the variations were small (see particularly Subject 83). Samples a and b in Subjects 70 and 71 were analyzed at the same time as those in Subject 69 and showed little or no variation.

In Subject 7 there is an apparent trend toward decreasing values throughout the 10 month period during which he was studied. There was no known change in the subject's physical condition, diet, or environment during this time.<sup>3</sup> No definite trend in either direction is evident in the other subjects on whom more than two or three determinations were carried out.

It appears from the data presented in Table I that in most, if not all, persons in health the concentration of cholesterol in the blood serum is maintained at a constitutional level which is characteristic for each individual and from which large deviations do not ordinarily occur. The range of variation in a given person over considerable periods of time is far less than the variation among different persons. It is impossible, therefore, to state on the basis of a single determination that the total cholesterol concentration of the serum is "normal" in a particular person. It may be within the normal range of variation but still be abnormal for that individual. Thus a value of 340 mg. per 100 cc., which is apparently normal for Subject 61, would be quite abnormal for Subject 6. The use of the term "normal concentration of total cholesterol in the serum" in the customary general sense is without definite meaning.

#### SUMMARY

The concentration of total cholesterol in the blood serum was determined two or more times at varying intervals, up to 28

<sup>3</sup> In a sample of serum analyzed after submission of this paper for publication (31 months after the first analysis in Subject 7) the total cholesterol concentration was 270.9 mg. per 100 cc.; i.e., almost exactly the same as the value found in the first sample (269.0 mg.).

months, in twenty-five healthy adult persons. The result leads to the conclusion that the variation in a given individual over considerable periods of time is much less than the variation among individuals. In most, if not all, persons in health the amount of cholesterol in the serum appears to be maintained at a constitutional level which is characteristic for each individual and from which large deviations do not ordinarily occur.

*Addendum*—In a recent paper (7), which came to the author's attention after submission of this manuscript for publication, Gildea, Kahn, and Man noted as an incidental finding that the general level of cholesterol and total fatty acid concentrations in the serum remained relatively constant in eight normal subjects over periods of 1 to 3 years. They concluded "that high or low or average lipoids represent a fundamental characteristic of an individual's constitutional makeup," *i.e.*, essentially the same conclusion as that reached in the present investigation in regard to cholesterol.

The principal conclusion of the Yale workers was that the serum lipids were consistently higher in men of pyknic (stocky) body build than in men of leptosomic (slender) physique. Though considerable attention has been paid to the possibility that there is a relation between body build and serum cholesterol concentration, it has been impossible to make out any such relationship in the persons studied in this laboratory. Several of the highest values occurred in subjects of slender build while, conversely, values well below the average were seen frequently in stocky persons. It must be admitted, however, that the subjects were not classified by a trained observer.

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# THE APPARENT CREATININE OF SERUM AND LAKED BLOOD ULTRAFILTRATES

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Among the numerous filtrates which have been utilized in the study of blood creatinine the ultrafiltrate is of special interest for several reasons. In the first place, it is generally held that ultrafiltration itself produces no chemical changes, so one might expect an ultrafiltrate to contain the blood creatinine substances in their original state, unless they are unstable. Also, an ultrafiltrate is akin to the glomerular filtrate, and the results should be of some interest in connection with the origin of urinary creatinine. Less intriguing, and more certain, is the fact that ultrafiltration is a means of deproteinizing without diluting—a highly important consideration if one attempts to precipitate a non-protein constituent.

In vividiffusion experiments, Abel, Rowntree, and Turner (1) noted that the original dialysate gave reactions for creatinine. Hunter and Campbell (2) and Plass (3) found the creatinine values in maternal and cord plasma essentially the same. Achard, Lévy, and Potop (4) reported that serum and its ultrafiltrate have the same creatinine content, as determined by the original picric acid method of Folin (5), in the case of normal human subjects, normal dogs, and human cases of retention. Confirmation of this finding by other methods has been reported (6, 7). The writer has already reported that the apparent creatinine of ultrafiltrates of serum from human subjects and dogs with retention is precipitable with picric acid and potassium chloride at room temperature, and that using rubidium chloride, as suggested by studies of known creatinine by Greenwald and Gross (8), makes it possible to precipitate most of the apparent creatinine of normal serum ultra-

filtrates at low temperatures (9, 10). Under conditions which differed from those originally employed by Behre and Benedict with picric acid filtrates, Danielson (7) found that the apparent creatinine of oxalate plasma ultrafiltrates could be removed with kaolin, and resembled true creatinine in other respects. Benedict and Behre (11) found that the color reaction which diluted ultrafiltrates of human plasma and beef serum, as well as blood filtrates, give with 3,5-dinitrobenzoate is different from that of creatinine.

To facilitate isolation, we attempted to reduce the amount of rubidium chloride and picric acid used in precipitations to the point where rubidium picrate and excess of picric acid would not precipitate, so that purification of the chromogenic precipitate might be as simple, and the result as significant, as possible. As this point was approached, precipitation of apparent creatinine failed in the case of normal dog serum ultrafiltrates, while creatinine added before or after ultrafiltration, or to a synthetic ultrafiltrate,<sup>1</sup> was still so completely precipitated that only a few hundredths of a mg. per 100 cc. remained in solution. Also, if whole blood was laked with 4 volumes of water, and the ultrafiltrate of this saturated with picric acid, kaolin experiments on the laked blood ultrafiltrate yielded essentially the same results which Behre and Benedict (12) originally obtained with picric acid filtrates of whole blood. The use of ultrafiltrates has thus become of interest in both of the essential phases of this problem,—differentiating between blood creatinine and added creatinine, and obtaining blood creatinine or its degradation products for purposes of study.

#### EXPERIMENTAL

##### *Precipitation of Apparent Creatinine*

*Procedure*—15 cc. of serum were ultrafiltered overnight through No. 300 cellophane in a Nicholas ultrafilter (13), under a nitrogen pressure of 400 pounds per sq. inch. The chromium-plated filter was lightly paraffined by heating the parts in a drying oven and dipping them in a chloroform solution of paraffin. A few drops of toluene were added to the serum and the collecting beaker, and during hot weather the filter was enclosed in a fiber carton containing a piece of dry ice. Evaporation of the ultrafiltrate was

<sup>1</sup> See foot-note 4.

prevented by a sponge rubber gasket between the bottom of the filter and the rim of the collecting beaker.

10 cc. of ultrafiltrate were shaken to saturation with 250 mg. of picric acid at room temperature and the tube was then placed in the refrigerator for an hour to precipitate excess picric acid.<sup>2</sup> After centrifugation, the supernatant liquid was decanted into another tube and mixed at once after addition of 0.15 cc. of 10 per cent rubidium chloride. Refrigeration at 5–7° was then resumed. Initial and later determinations of apparent creatinine were made by diluting 1 cc. of the supernatant liquid with 4 cc. of 1.2 per cent picric acid,<sup>3</sup> adding 0.25 cc. of 10 per cent sodium hydroxide, centrifuging during color development, and reading promptly 10 minutes after addition of the alkali. The standards contained 0.3 to 0.6 mg. of creatinine per 100 cc. of 1.2 per cent picric acid. The practise of using empirical curves in the calculation of results, which was introduced by Hunter and Campbell (15), was again followed, as in a preceding study (16).

Approximate determinations of the apparent creatinine in the precipitates were made by suspending them in either 5 or 10 cc. of 1.2 per cent picric acid, adding 0.25 or 0.50 cc. of 10 per cent sodium hydroxide, shaking thoroughly for several minutes, and centrifuging. The color in this case may be read much later than 10 min-

<sup>2</sup> The purpose of this step is to reduce the picric acid concentration to a constant level, so that a determination of apparent creatinine at this point will be comparable with later ones. In the case of normal ultrafiltrates less than 0.05 mg. of apparent creatinine per 100 cc. was found in the precipitated picric acid. In moderate retention, a non-chromogenic picrate precipitate containing uric acid and basic substances was obtained at this point. In an extreme case of retention (32.2 mg. of apparent creatinine per 100 cc.) a granular precipitate like that from urine started to form even earlier while the ultrafiltrate was being saturated with picric acid. This precipitate contained over half of the chromogenic substance, and more precipitated during refrigeration.

<sup>3</sup> Undiluted ultrafiltrate, saturated with picric acid and made alkaline with sodium hydroxide, shows intense and prolonged color development. The diluted ultrafiltrate behaves nearly like Folin picric acid filtrates. Dilution with 1.2 per cent picric acid also equalizes the picric acid concentration in standard and unknown. When the excess of picric acid is titrated, the refrigerated ultrafiltrates were found to have retained 1.6 to 1.8 gm. per 100 cc. in solution, partly, of course, as sodium picrate. Besides sodium bicarbonate, the ultrafiltrates contain sodium chloride in amounts sufficient to increase the solubility of picric acid (14).



utes after addition of the alkali, for the chromogenic precipitate, unlike the supernatant liquid, behaves like true creatinine in this respect. This procedure is very sensitive, for 0.01 mg. of creatinine in 5 cc. of 1.2 per cent picric acid is readily determined, and if obtained from 10 cc. of ultrafiltrate, corresponds to 0.1 mg. per 100 cc.

TABLE I

*Showing Extent to Which Apparent Creatinine Can Be Precipitated from Ultrafiltrates of Normal Sera of Various Species*

| Subject         | Apparent creatinine, mg. per 100 cc. |                  |                |            |                  | Days<br>required<br>for precipi-<br>tation |
|-----------------|--------------------------------------|------------------|----------------|------------|------------------|--|
|                 | Serum                                | Ultrafiltrate    |                |            | Found<br>in ppt. |  |
|                 |                                      | Initial<br>value | Final<br>value | Difference |                  |  |
|                 | (1)                                  | (2)              | (3)            | (4)        | (5)              |  |
| Dog 1           | 1.55                                 | 1.28             | 0.50           | 0.78       | 0.71             | 4  |
| " 4             |                                      | 1.56             | 0.75           | 0.81       | 0.76             | 6  |
| " 4             | 1.70                                 | 1.38             | 0.53           | 0.85       | 0.67             | 6  |
| " 4             | 1.85                                 | 1.55             | 0.50           | 1.05       | 1.23             | 2  |
| " 8             |                                      | 1.65             | 0.50           | 1.15       | 1.01             | 3  |
| " 8             | 1.65                                 | 1.22             | 0.62           | 0.60       | 0.65             | 3  |
| " 12            | 1.70                                 | 1.17             | 0.50           | 0.67       | 0.52             | 7  |
| " S             |                                      | 1.65             | 0.62           | 1.03       | 0.98             | 2  |
| " X             | 1.50                                 | 1.04             | 0.53           | 0.51       | 0.46             | 5  |
| Beef            | 2.27                                 | 2.17             | 0.58           | 1.59       | 1.51             | 6  |
| "               | 2.45                                 | 2.20             | 0.50           | 1.70       | 1.82             | 2  |
| "               | 2.00                                 | 1.90             | 0.90           | 1.00       | 1.09             | 2  |
| Pig             | 2.50                                 | 1.90             | 0.73           | 1.17       | 1.08             | 3  |
| "               |                                      | 1.30             | 0.75           | 0.55       | 0.59             | 8  |
| Human, O. H. G. | 1.38                                 | 1.04             | 0.37           | 0.67       | 0.52             | 2  |
| " mixed         | 1.32                                 | 1.08             | 0.38           | 0.70       | 0.72             | 3  |
| " L. D. A.      | 1.50                                 | 1.50             | 0.58           | 0.92       | 0.91             | 3  |

*Results*—The above procedure was applied to sera obtained from normal dogs by venepuncture during the postabsorptive period, to human sera obtained before the noon meal, and to sera of beef and pig bloods centrifuged within an hour after the animals were stuck. The results are shown in Table I. The "creatinine" content of the sera was determined by the original Folin (5) method, since ultrafiltrates saturated with picric acid were to be used in the remaining determinations. The initial value in the

ultrafiltrate was determined at 1:5 dilution in the manner already described, after precipitation of the excess of picric acid by refrigeration. The amount of apparent creatinine remaining in solution was determined daily or every 2nd day in the same manner. The final value subtracted from the initial value gives a difference which is about equal to the apparent creatinine found in the precipitate. Values for the latter are corrected for withdrawal of samples for analysis. The time required for precipitation, as shown in the last column of Table I, is frequently longer than was stated in the preliminary report (10).

The "final value" after precipitation (Table I) is quite large in some sera, and would be definitely larger throughout if color due to sodium picrate were not excluded by using empirical curves in calculating the results. Neither creatinine nor glucose can account for this value, but the latter may account for the fact that the color in these determinations continues to develop after 10 minutes and ultimately changes to brown. From synthetic ultrafiltrates<sup>4</sup> containing the same amount of salts, urea, and glucose as serum and sufficient glycine to equal all of the amino acid nitrogen, 1 or 2 mg. of creatinine per 100 cc. are precipitated by this procedure almost completely. In such experiments the undiluted supernatant liquid, if made alkaline with sodium hydroxide, only develops as much color in 10 minutes as is equivalent to 0.1 mg. of creatinine per 100 cc. This is mainly due to glucose and substances other than creatinine in the synthetic ultrafiltrate. The much larger amount of unprecipitable material yielding color in the case of serum ultrafiltrates is probably due to the presence of substances which are "chromogenic" only because a large concentration of picrate is used in these determinations.

The chromogenic substance which is precipitated continues to match a creatinine standard at the same depth much longer than 10 minutes. It has not yet been obtained as a pure double picrate from either normal or retention serum ultrafiltrates. Its precipi-

<sup>4</sup> Dissolve 0.477 gm. of KCl, 2.52 gm. of NaHCO<sub>3</sub>, 0.134 gm. of NaH<sub>2</sub>PO<sub>4</sub> · H<sub>2</sub>O, 0.059 gm. of Na<sub>2</sub>SO<sub>4</sub>, 6.28 gm. of NaCl, 1 gm. of glucose, 0.4 gm. of urea, and 0.375 gm. of glycine in water. Dissolve 0.250 gm. of CaCO<sub>3</sub> in 50 cc. of 0.1 N HCl, and add it to the above solution. Also add 0.0498 gm. of MgO, dissolved in 24.8 cc. of 0.1 N HCl. Pass CO<sub>2</sub> through the solution until it clears, and dilute to 1 liter.

tation from normal serum ultrafiltrates is not to be regarded as an adsorption by, or entrainment in, the excess of rubidium picrate, for the latter precipitates mainly in the first 24 hours. The precipitation of apparent creatinine has often not begun at this time, and may require 6 days or more to go to completion. During this time the precipitated rubidium picrate changes in color from yellow to orange, and if the crystals are then immersed in alkaline picrate on a spot plate they become bright red, lose a coating of material giving the Jaffe reaction, and are pale yellow once more. Thus it appears that rubidium picrate furnishes a suitable surface on which the precipitation of the chromogenic substance in normal ultrafiltrates occurs.

The amount of rubidium required varies widely, depending on the source of the serum and the purpose of the experiment. To precipitate added creatinine, or apparent creatinine of retention serum or beef serum ultrafiltrates, requires only one-fifth of the amount mentioned in the procedure. In fact added creatinine and apparent creatinine of retention serum can be precipitated at room temperature with potassium (9), and in some cases by saturating the ultrafiltrate with picric acid and refrigerating without adding either rubidium or more potassium than is already present.

#### *Source of Chromogenic Precipitate*

The procedure which has been described is not open to theoretical objections which apply when a partly soluble adsorbent such as Lloyd's reagent is used. Nothing has been introduced in the interval between the determinations before and after precipitation, and the precipitate, if necessary, can be dissolved completely. The most obvious objection to the procedure is that the ultrafiltrates, saturated with picric acid, are allowed to stand, although refrigerated. Picric acid filtrates of whole blood are known to deepen if allowed to stand. Also, the acidity of the saturated ultrafiltrates might convert a non-chromogenic creatinine-like substance to a precipitable chromogenic one.

In Table II it is shown that the apparent creatinine content of normal dog serum ultrafiltrates, which were saturated with picric acid and refrigerated without addition of rubidium chloride, remained essentially unchanged for 10 days. Still longer periods

are covered in some of the controls of experiments in a subsequent section. The ultrafiltrate which has stood is, however, always diluted with 4 volumes of pure 1.2 per cent picric acid before addition of alkali.

A small amount of precipitate appeared in the experiments recorded in Table II, but this contained no appreciable amount of chromogenic substance. In some cases not included in Table I, in which rubidium was added, the apparent creatinine content also remained constant for a week. The precipitate of rubidium and other picrate was non-chromogenic, and the filtrate from this, on further refrigeration, decreased in apparent creatinine content and deposited a few highly chromogenic crystals. Finally 240 cc. of

TABLE II

*Showing That Apparent Creatinine Content of Dog Serum Ultrafiltrates Saturated with Picric Acid and Refrigerated Remains Constant for a Long Period*

The figures represent mg. of creatinine per 100 cc. of blood.

| Serum | Serum ultrafiltrate, saturated with picric acid and refrigerated for indicated time |         |         |          |          |          |          |          |          |          |
|-------|---|---------|---------|----------|----------|----------|----------|----------|----------|----------|
|       | 0 hr.   | 48 hrs. | 96 hrs. | 120 hrs. | 144 hrs. | 168 hrs. | 192 hrs. | 216 hrs. | 240 hrs. | 264 hrs. |
| 1 60  | 1.15  | 1.20    | 1.32    |          | 1.20     |          |          |          | 1.20     |          |
| 1 65  | 1.25  | 1.35    |         |          | 1.28     |          |          | 1.20     | 1.25     |          |
| 1.37  | 1.17  | 1.27    |         |          | 1.20     |          | 1.21     |          |          |          |
| 1.57  | 1.17  |         | 1.20    |          |          | 1.10     |          |          |          | 1.20     |
| 1.65  | 1.22  | 1.30    |         | 1.17     |          |          |          |          |          | 1.25     |

collodion ultrafiltrate from normal dog serum were divided into four portions, with each of which the precipitation was carried out as described. The precipitation fortunately proceeded at a different rate in the four lots of the same ultrafiltrate. From the decrease in apparent creatinine it was calculated that the total amounts in the precipitates should be 0.31, 0.15, 0.23, and 0.39 mg. The precipitates, completely dissolved, contained 0.30, 0.16, 0.26, and 0.32 mg. These observations, as well as those in Table I, suggest that the chromogenic substance in the ultrafiltrate is the source of the chromogenic substance in the precipitate, whether it decomposes and precipitates, or precipitates unchanged. In the case of retention sera there was of course no doubt concerning this,

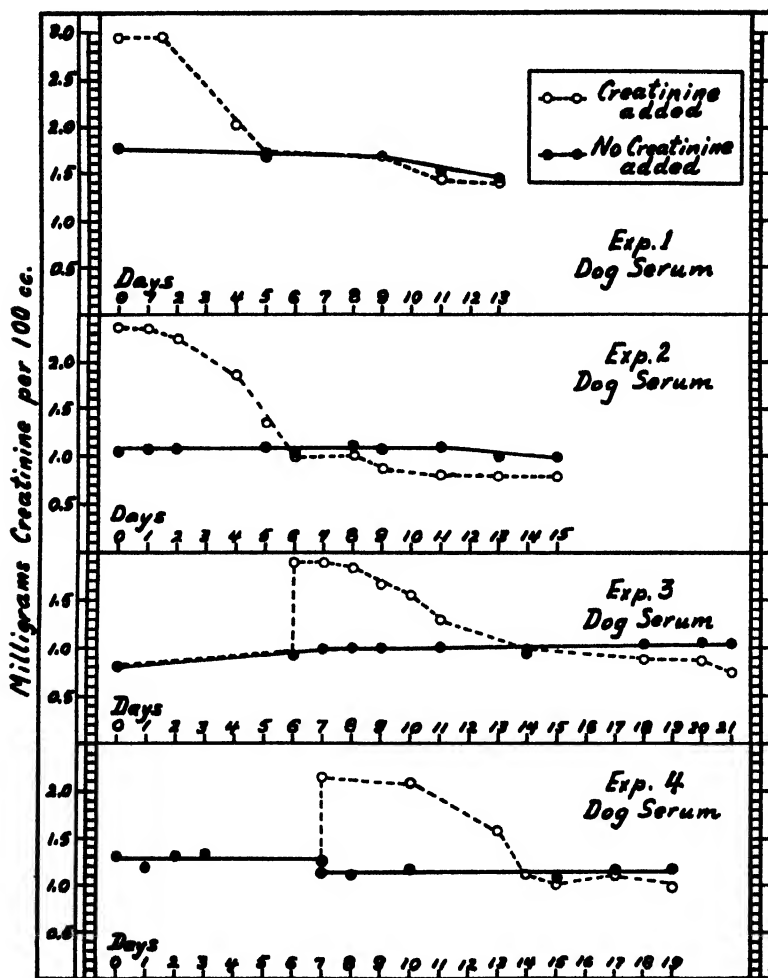


FIG 1. Precipitation of added creatinine from dog serum ultrafiltrate without precipitation of apparent creatinine. The offset in the control of Experiment 4 is due to manipulations.

since the amount of chromogenic substance was so large, and its precipitation so rapid.

#### *Is Ultrafiltrate Creatinine True Creatinine?*

**Precipitation Experiments**—In the remainder of this study collodion membranes were used. The vacuum produced by an

ordinary water pump replaced the high nitrogen pressure required with cellophane. The filtering surface was 4.5 inches in diameter, and the yield 200 cc. in 2 hours from 300 cc. of serum. The ultrafilter was a modification of one described by Wilson and Holiday (17) and the 2 per cent parlodion solution in alcohol-ether-acetic acid was prepared as described by these authors. 10 cc. of this solution were allowed to flow upon a 6 inch plate glass disk floating on mercury in a desiccator containing sulfuric acid. No excess was drained off. The drying time preceding immersion of the glass disk and its membrane in water was 23 minutes. Calcium phosphate precipitated in the ultrafiltrates under a vacuum, and was removed by filtration. In hot weather the receiving vessel was placed in ice. If the froth obtained on shaking the ultrafiltrate did not immediately disappear, the ultrafiltrate was rapidly refiltered through another membrane.

Large lots of dog serum or beef serum were divided into two lots, to one of which creatinine was added before ultrafiltration. In some instances an ultrafiltrate was divided into two lots, and creatinine added to one. In each 100 cc. of ultrafiltrate 1.7 gm. of picric acid were dissolved. This left the ultrafiltrate clear, hence free of protein. After addition of 0.3 cc. of 10 per cent rubidium chloride (one-fifth the amount used in experiments recorded in Table I) the solution was shaken immediately, and was then refrigerated at 5-7°.

The results are shown in Fig. 1. In the case of dog serum the distinction between apparent creatinine and added creatinine is seen at a glance, for the added creatinine precipitates readily even with this smaller amount of rubidium, while apparent creatinine does not.<sup>5</sup> Details of the experiments are given in the following protocols.

<sup>5</sup> Differentiation of apparent creatinine from true creatinine by an analogous precipitation method had already been accomplished by Behre and Benedict (private communication) before these experiments were completed. The fact that apparent creatinine of dog serum ultrafiltrates remains in solution for such long periods of time seemed to us an additional point of interest. It makes it difficult to assume that apparent creatinine decomposes in picric acid solution into true creatinine, and that the latter precipitates. Precipitation fails for as long as 15 days under conditions where any creatinine formed should precipitate. Apparent creatinine can then be precipitated within a few days by doubling the amount of rubidium chloride, which could hardly effect decomposition. We have considerable

*Experiment 1*—Creatinine (1.33 mg. per 100 cc.) was added to half of the serum before ultrafiltration. In the control ultrafiltrate the apparent creatinine precipitated very slowly, 0.33 mg. per 100 cc. in 13 days. The precipitate contained 0.40 mg. per 100 cc. The added creatinine of the other ultrafiltrate precipitated in 5 days, after which the two ultrafiltrates showed the same values.

*Experiment 2*—The control ultrafiltrate remained unchanged, if a minute decrease in apparent creatinine at the end of the experiment is disregarded. No chromogenic substance was found by filtration; in fact, no visible precipitate appeared. Creatinine added to the other portion of this serum precipitated rapidly from the ultrafiltrate, and very slow precipitation continued. The precipitate contained 1.63 mg. of creatinine per 100 cc. of ultrafiltrate, which exceeded the 1.33 mg. per cent added by the amount that precipitation passed the control level.

*Experiment 3*—In this ultrafiltrate the apparent creatinine value was only half as large as in Experiment 1, and rose very slowly. No chromogenic precipitate was recovered from the control at the end of the experiment. From a separate portion of the same ultrafiltrate creatinine added on the 6th day (1 mg. per 100 cc.) precipitated in 8 days.

*Experiment 4*—After the apparent creatinine content had remained unchanged for 7 days, the ultrafiltrate was filtered. To half of it was added 1 mg. of creatinine per 100 cc. To the other half an equal amount of 0.1 N hydrochloric acid was added, and the determination of the control value repeated. The added creatinine precipitated in 7 days, and very slight precipitation of apparent creatinine also occurred. Analysis of the precipitate confirmed this. It appears that added creatinine may supply the picrate on which a slight precipitation of apparent creatinine occurs. The control ultrafiltrate never developed a precipitate.

*Note*—For all additions of creatinine the stock solution containing 1 mg. per cc. in 0.1 N hydrochloric acid was used.

In the case of beef serum ultrafiltrates, the same technique did not distinguish clearly between added creatinine and apparent

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evidence that the chromogenicity of our precipitate is due to creatinine, but for the above reasons are examining the precipitate for substances with which the creatinine could have been combined.

creatinine, for both precipitated (Fig. 2, Experiment 5). This still occurred when addition of rubidium or more potassium than was already present was omitted, but quite slowly (Experiment 6). The creatinine added to the serum precipitated in 4 days, at which time precipitation of apparent creatinine had not begun.

*Kaolin Experiments*—With a series of normal bloods from the indicated sources the kaolin experiment of Behre and Benedict (12) was carried out. The results are shown in Columns 1 and 2 of Table III. The corresponding experiments with creatinine added to blood were also carried out and proved that the kaolin used was satisfactory for the removal, from Folin picric acid

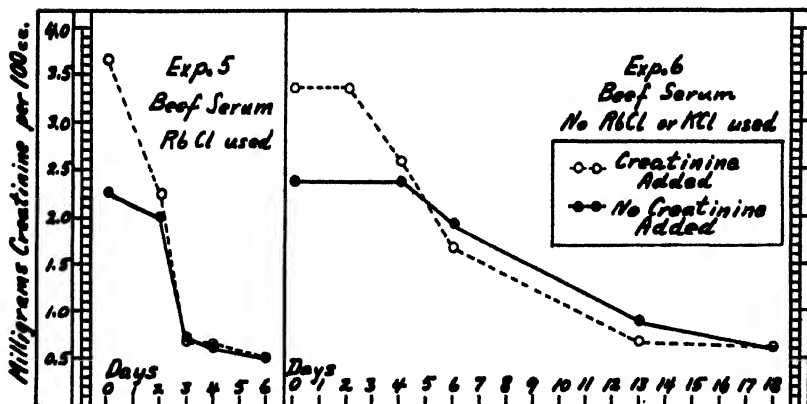


FIG. 2. Precipitation of added and apparent creatinine from beef serum ultrafiltrate, with and without the use of rubidium chloride.

filtrates, of creatinine added to blood. Samples of the same bloods were laked with 4 volumes of water and ultrafiltered. The ultrafiltrates were saturated with picric acid and shaken for 10 minutes with 2 gm. of kaolin per 25 cc. This removed only small and irregular amounts of apparent creatinine (Column 6). But creatinine added to the same bloods was removed by kaolin under these conditions (Column 9). In the case of dog blood the addition of 1 mg. per cent of creatinine increased the observed creatinine content by 1.10 mg. per cent, as shown in the averages of Columns 4 and 7. Subtracting the average of Column 6 from that of Column 9 gives the observed value for added creatinine removed as 1.07 mg. per cent.



Suitable kaolin was difficult to obtain. The sample finally used was Eimer and Amend's acid-washed, American Standard, and was the best of six lots from as many sources. It was, however, whiter and much more finely divided than the product formerly used, and tended to remove "creatinine." Shaking 1.2 per cent

TABLE III

*Showing That Kaolin Removes Creatinine Added to Blood and Recovered in Laked Blood Ultrafiltrates, but Removes Very Little Apparent Creatinine from Laked Blood Ultrafiltrates*

The figures represent mg. of creatinine per 100 cc. of blood.

| Source of blood | Without added creatinine   |                     |                   |                           |                     |                   | With 1 mg. creatinine per 100 cc. added. Laked blood ultrafiltrate |              |            |
|-----------------|----------------------------|---------------------|-------------------|---------------------------|---------------------|-------------------|--|--------------|------------|
|                 | Folin picric acid filtrate |                     |                   | Laked blood ultrafiltrate |                     |                   | Before kaolin  | After kaolin | Difference |
|                 | Before kaolin<br>(1)       | After kaolin<br>(2) | Difference<br>(3) | Before kaolin<br>(4)      | After kaolin<br>(5) | Difference<br>(6) |  |              |            |
| Dog             | 2.20                       |                     |                   | 2.65                      | 2.62                | -0.03             | 3.50   | 2.62         | -0.88      |
| "               | 2.00                       |                     |                   | 2.27                      | 2.05                | -0.22             | 3.12   | 1.90         | -1.22      |
| "               | 2.45                       |                     |                   | 2.52                      | 1.97                | -0.55             | 3.65   | 1.92         | -1.73      |
| "               | 2.02                       |                     |                   | 2.05                      | 1.82                | -0.23             | 3.27   | 2.00         | -1.27      |
| "               | 2.47                       |                     |                   | 2.67                      | 2.52                | -0.15             | 4.00   | 2.57         | -1.43      |
| "               | 2.00                       | 1.72                | -0.28             | 2.32                      | 2.07                | -0.25             | 3.47   | 2.05         | -1.42      |
| "               | 2.17                       | 1.95                | -0.22             | 2.87                      | 2.27                | -0.60             | 4.00   | 2.30         | -1.70      |
| "               | 2.20                       | 1.77                | -0.43             | 3.00                      | 2.37                | -0.63             | 4.00   | 2.60         | -1.40      |
| "               | 2.00                       |                     |                   | 2.20                      | 1.80                | -0.40             | 3.25   | 1.87         | -1.38      |
| "               | 2.25                       | 2.15                | -0.10             | 2.90                      | 2.90                | 0.00              | 4.20   | 2.87         | -1.33      |
| Average.....    |                            |                     |                   | 2.55                      | 2.24                | -0.31             | 3.65   | 2.27         | -1.38      |
| Human           | 2.10                       | 1.90                | -0.20             | 2.22                      | 2.00                | -0.22             | 3.75   | 2.52         | -1.23      |
| "               | 2.45                       | 2.37                | -0.08             | 2.65                      | 2.45                | -0.20             | 3.92   | 2.50         | -1.42      |
| "               | 3.12                       | 3.12                | 0.00              | 3.37                      | 3.40                | +0.03             | 4.80   | 3.72         | -1.08      |
| "               | 2.87                       | 2.70                | -0.17             | 3.35                      | 3.35                | 0.00              | 5.00   | 3.72         | -1.28      |
| Average.....    |                            |                     | -0.11             | 2.90                      | 2.80                | -0.10             | 4.37   | 3.11         | -1.26      |

picric acid for 10 minutes with 2 gm. of this kaolin per 25 cc. of picric acid did not alter the amount of color which the picric acid would subsequently produce with creatinine. Standards containing 0.3 mg. per cent of creatinine, actual concentration, prepared with picric acid treated or untreated with kaolin matched perfectly, contrary to the experience of Hayman, Johnston, and

Bender (6). Our technique differs from theirs in the source of kaolin and the fact that it was removed by centrifuging. We do not object to filtration when the total amount of color dealt with is as large as in the actual application of this experiment.

The values in Column 1 of Table III are rather high for normal bloods. In an attempt to secure greater uniformity all determinations of Table III were read against standards containing 0.6 mg. of creatinine per 100 cc. We confirmed the finding of Hunter and Campbell (15) that this increases the value for normal blood, even though empirical curves are used in calculating results with the 0.3 and 0.6 mg. standards. Also, the room temperature during this part of the study was rarely below 30°, and, as the temperature increases, the picric acid concentration in saturated blood filtrates increases while that in the standard is kept fixed.

#### DISCUSSION

The present study, as well as the data in the literature, are as yet too incomplete to answer the question whether the substance precipitated from normal serum ultrafiltrates is the same as that eluted from Lloyd's reagent or kaolin<sup>6</sup> with which normal whole blood filtrates have been shaken. At the end of complex isolation procedures the substance eluted from Lloyd's reagent has been identified as creatinine only in the case of picric (19) and tungstic acid (20) filtrates of beef blood and tungstic acid filtrates of dog blood (16). Data on its rate of color development are available in the case of trichloroacetic acid filtrates of human serum (6). One report is available in which comparative determinations of the eluted substance are made by the picrate and 3,5-dinitrobenzoate methods (21). So far none of the findings has established a difference between the eluted substance and creatinine.

<sup>6</sup> Although kaolin appears to remove no "creatinine" from picric acid filtrates of whole blood, chromogenic substance can be eluted from the kaolin after shaking the filtrates with it (18). This observation has also been made, though differently interpreted, in the case of serum (6). Since the substance eluted from Lloyd's reagent is removed from picric acid filtrates of normal blood by kaolin (16), the writer assumes that it is the same as the substance eluted from kaolin. The unexplained anomaly that this substance appears to become chromogenic in the process of adsorption and elution is at present the only differential point between it and the substance precipitated from serum ultrafiltrates.

But a conclusion can well be postponed until reasonably complete data are available for blood and serum of at least a few species in which the supply is adequate for all purposes including isolation. For this reason we are continuing a study of the nature of the chromogenic substance precipitable from dog and beef serum ultrafiltrates—the same species of which whole blood was previously examined by means of the Lloyd's reagent procedure.

In the case of advanced retention, a similarity between isolation findings by the two procedures in question is of course expected. It has never been implied that the huge amount of chromogenic substance eluted from Lloyd's reagent in such cases originated in the process of adsorption and elution. Creatinine was first isolated from retention blood by means of an adsorbent soluble in alkaline picrate solution (19), so that chromogenicity could be traced. The possibility that creatinine is not present in retention blood rests entirely on the supposition that decomposition or transformation could occur in the isolation process. For this no extensive evidence has been presented to date. But the present finding that there is present in serum ultrafiltrates a chromogenic substance simulating creatinine in its precipitation behavior (Table I), yet not identical with it (Fig. 1), may well urge caution in concluding that creatinine itself accounts for nearly all of the apparent creatinine of retention blood, at least in the case of the dog. Presumably a larger concentration of the same substance would precipitate more rapidly.

For large scale preparation we dissolve 1.7 gm. of picric acid per 100 cc. in beef serum ultrafiltrate by stirring at room temperature, add 0.3 cc. of 10 per cent rubidium chloride, and refrigerate at 5° until analysis of the supernatant liquid indicates that precipitation has reached profitable limits. In nine large scale precipitations which have yielded picrate containing apparent creatinine equivalent to 30 mg. of true creatinine in color production, the longest refrigeration was 5 days. As shown in Fig. 1, the apparent creatinine of dog serum ultrafiltrate does not precipitate under the above conditions, but twice the amount of rubidium chloride does precipitate it from our large scale collodion ultrafiltrates. The precipitate, however, contains so much more rubidium picrate than chromogenic picrate that separation by recrystallization alone may be impossible. Recrystallizations in the presence of

excess rubidium picrate cause only minute losses of chromogenic substance or substances. The chromogenic precipitate can be washed with a mixture of 1 part alcohol and 3 parts peroxide-free ether to remove picric acid without notable loss of chromogenic substance, which is, however, extracted from the precipitate by acetone, and more easily with warm water.

The total amount of apparent creatinine that can be precipitated on a small scale from cellophane ultrafiltrates with large amounts of rubidium is shown in Column 5 of Table I. The amounts which are precipitated vary considerably even in one species, such as the dog, and are far greater than any that should escape precipitation with smaller amounts of rubidium, or detection in kaolin experiments, were they present as true creatinine. The same point has already been considered in connection with the chromogenic substance eluted from Lloyd's reagent (16).

In the early stages of this study the thought was entertained, though not expressed, that creatinine itself might be present in serum ultrafiltrates. The diverse findings with kaolin in different filtrates, and the excretion of creatinine in urine, could thus be considered as so many evidences of decomposition of the chromogenic substance in blood. The present experiments have done little to advance this simple theory. Evidently removal of the proteins of laked blood by ultrafiltration does not result in any instability of "creatinine" demonstrable with kaolin, and the precipitation of apparent creatinine from serum ultrafiltrates is materially more difficult than that of creatinine. The experiments have, however, indicated that precipitation of the proteins with picric acid is not an indispensable feature of the kaolin experiment—a possibility which has been considered (7). With the present technique it was also possible to reduce the amount of picric acid with which the non-protein constituents came in contact. This amount was 0.5 gm. of picric acid per 25 cc. of laked blood ultrafiltrate, and the time of shaking was 10 minutes. The result of the modifications was a series of "creatinine" values that were between those obtained with the Folin method and the Myers method. Unfortunately we have not yet tested the modification of the kaolin procedure with beef blood. In beef serum the difficulty of differentiating between apparent and true creatinine appears to be greater than in other bloods, both in the precipitation

tests reported here, and in the case of the 3,5-dinitrobenzoate color reaction (11).

#### SUMMARY

1. A procedure is described for small scale precipitation of apparent creatinine from cellophane ultrafiltrates of sera of various species.

2. Large scale precipitations from collodion ultrafiltrates of beef and dog serum are described.

3. A part of the apparent creatinine of normal serum ultrafiltrates simulates creatinine in its precipitation behavior, is not identical with it, but may be related to it.

4. The chromogenic picrate precipitated from normal and retention serum ultrafiltrates has not as yet been obtained in pure form for identification.

5. The procedures used are highly sensitive for the precipitation of added creatinine from serum ultrafiltrates or other complex solutions, but are not specific for it.

6. In the case of normal dog blood and normal human blood the apparent creatinine of laked blood ultrafiltrates is not removable by kaolin under the conditions described by Behre and Benedict, while added creatinine is removable.

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# EXPERIMENTS ON THE PRECIPITATION OF CREATININE RUBIDIUM PICRATE FROM BLOOD PLASMA FILTRATES

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In 1924 Greenwald and Gross (1) reported studies on the preparation and solubility of creatinine rubidium picrate and suggested that, since this salt has a lower solubility than the corresponding potassium salt, it might prove useful in the precipitation of creatinine from blood. Recently Gaebler (2) reported briefly the precipitation, by means of picric acid and rubidium chloride, of about 60 per cent of the creatinine chromogenic material present in undiluted ultrafiltrates from normal dog sera, which contained from 1.22 to 1.65 mg. per cent of the material. Gaebler reported no experiments to show the behavior of pure creatinine solutions under similar conditions nor did he describe the details of the technique employed,<sup>1</sup> but he recorded the very important finding that the solubility of creatinine rubidium picrate is greatly decreased if the precipitation is carried out in a refrigerator.

The work reported in the present paper was undertaken with the hope that by the use of rubidium chloride and picric acid a considerable amount of the blood chromogenic material could be precipitated, and that this precipitate could then be decomposed with hydrochloric acid, and, after removal of the picric acid by benzene, tested for a typical creatinine reaction with dinitrobenzoic acid<sup>2</sup> (3). Using a procedure, the details of which

<sup>1</sup> In a personal communication, which we received after the present work had been begun, Dr. Gaebler has discussed his procedure in detail with us.

<sup>2</sup> It may be pointed out here that, since many compounds other than creatinine will form insoluble picrates, the precipitation of the blood chromogenic material as a picrate does not serve to identify the chromogenic material as creatinine.



will be described below, we were unable to precipitate any appreciable amount of the chromogenic material from plasma filtrates of several different species, although by the same procedure creatinine, whether in pure solution or added to the filtrates, was practically quantitatively precipitated as the double rubidium picrate. While we have thus not succeeded in carrying out the original object of the present work, we are reporting the results obtained because they appear to offer another striking example of the difference in behavior between the blood chromogenic material and true creatinine.

The procedure which we employed for the precipitation of creatinine consisted in the addition of small amounts of a 1 per cent solution of rubidium chloride (containing up to 30 times the amount of rubidium theoretically necessary to combine with the creatinine present) to solutions of creatinine in saturated picric acid. The walls of the containers were scratched until turbidity appeared and the solutions allowed to stand at refrigerator temperature (2-4°) for varying lengths of time, after which the supernatant liquid was filtered in the refrigerator through chilled filter paper, the filtrate resaturated with picric acid at room temperature, and its creatinine content determined by the Folin method (4).

By this procedure practically all of the creatinine is precipitated from pure solutions containing from 0.9 to 5 mg. per cent of the compound. The completeness of precipitation depends, within certain limits, upon the amount of rubidium present, the time of standing, and the concentration of creatinine in the original solution. All except about 0.2 to 0.25 mg. per cent of the creatinine is precipitated from a 0.5 mg. per cent solution after 72 hours if 30 times the theoretical amount of rubidium is used. From 0.9 mg. per cent creatinine solution, all except 0.2 mg. per cent is precipitated after 72 hours when 10 times the theoretical amount of rubidium is used. Stronger solutions of creatinine require less rubidium and shorter periods for complete precipitation.

The precipitated creatinine was liberated by treating the crystals which remained in the flask after decantation of the supernatant liquid, and those on the filter paper, with about 3 cc. of 3 N HCl.

After thorough stirring, the mixture was decanted into a centrifuge tube, centrifuged, and the supernatant liquid was poured into a small, accurate graduate. The treatment of the crystals with acid was repeated, with 3 or 4 cc. of  $N$  HCl, and the second supernatant fluid was added to the first. The clear, acid solution thus obtained was made to a small, definite volume and the picric acid completely removed by shaking with several (usually three) portions of benzene in a separatory funnel. The perfectly clear, colorless solution was then neutralized with sodium hydroxide, further diluted, and the creatinine determined by the Folin-Wu and dinitrobenzoic acid methods. From 80 to 100 per cent recoveries of precipitated creatinine were obtained in this way.

It was our original intention to apply the same precipitation procedure to plasma ultrafiltrates, saturated with picric acid, and two such filtrates were made with a Giemsa type ultrafilter (in which parlodion is used as the membrane). To obtain an undiluted filtrate with this filter it is necessary to discard at least the first 15 to 17 cc. of filtrate, and much time and material are required. It was found that a very satisfactory, undiluted, picric acid filtrate could be easily obtained with the use of much less material by shaking the plasma directly with solid picric acid. The plasma was first shaken in a shaking machine for about 20 minutes with 1 or 1.5 gm. of picric acid for each 50 cc. of plasma, after which the mixture was centrifuged and the supernatant fluid shaken with more picric acid (1 to 2 gm.) for a few hours. After sufficient shaking with picric acid a perfectly clear, picric acid-saturated liquid is obtained on centrifuging and filtering. The amount of picric acid and the time of shaking necessary for complete precipitation of protein are dependent upon the condition of the original plasma. Clear, relatively colorless plasma may give clear, saturated filtrates after shaking with picric acid for only about an hour, but plasma containing more protein may require 4 or 5 hours shaking with several additions of picric acid. By this method about 30 cc. of filtrate can be obtained from 50 cc. of plasma. The "creatinine" content of such plasma filtrates, determined by the Folin method, is practically identical with that of tungstomolybdate filtrates from the

| Sample No. | Source of plasma used | Tungstomolybdate filtrate, Folin-Wu determination | Rubidium precipitation of saturated picric acid filtrates |                               |                              |  |   |                |                       |
|------------|-----------------------|---|---|-------------------------------|------------------------------|--|---|----------------|-----------------------|
|            |                       |   | Creatinine added*   | Filtrate before precipitation | Filtrate after precipitation | Chromogenic plasma material precipitated | Per cent of added creatinine precipitated | Rubidium used† | Time of precipitation |
| 1          | Cat                   | 1.14  |   | 1.29                          | 1.30                         | 0  |   | 15             | 5 days                |
| 2          | Sheep                 | 1.07  |   | 0.98                          | 0.92                         | 0.06                                     |   | 10             | 65 hrs.               |
|            |                       |   | 1.0 (p.)  | 1.98                          | 0.90                         | 0.08                                     | 100                                       | 5              | 65 "                  |
|            |                       |   | 2.0   | 2.98                          | 0.89                         | 0.09                                     | 100                                       | 3.3            | 65 "                  |
| 3          | "                     | 1.18  |   | 1.10                          | 1.10                         | 0  |   | 18             | 72 "                  |
|            |                       |   | 1.0 (p.)  | 2.00                          | 1.06                         | 0  | 94  | 10             | 72 "                  |
| 4          | "                     | 2.04  |   | 1.69                          | 1.62                         | 0.07                                     |   | 10             | 76 "                  |
|            |                       |   | 1.0   | 2.69                          | 1.94                         | 0  | 75  | 10             | 76 "                  |
| 5          | "                     | 2.00  |   | 2.14                          | 2.05                         | 0.09                                     |   | 14             | 4 days                |
|            |                       |   |   | 2.14                          | 2.02                         | 0.12                                     |   | 30             | 4 "                   |
|            |                       |   | 1.0   | 3.14                          | 1.91                         | 0.23                                     | 100                                       | 8              | 4 "                   |
|            |                       |   | 1.0   | 3.14                          | 1.67                         | 0.47                                     | 100                                       | 10             | 4 "                   |
| 6          | "                     | 1.50  |   | 1.64                          | 1.54                         | 0.10                                     |   | 13             | 70 hrs.               |
|            |                       |   | 1.0   | 2.60                          | 1.46                         | 0.14                                     | 100                                       | 8              | 70 "                  |
| 7          | Beef (ultra-filtrate) | 1.13  |   | 1.28                          | 1.20                         | 0.06                                     |   | 8              | 41 "                  |
|            |                       |   | 2.0   | 2.28                          | 1.21                         | 0.05                                     | 100                                       | 4              | 41 "                  |
| 8          | Beef                  | 2.02  |   | 2.10                          | 1.95                         | 0.15                                     |   | 15             | 71 "                  |
|            |                       |   | 2.0 (p.)  | 4.00                          | 1.91                         | 0.09                                     | 100                                       | 7.5            | 71 "                  |
| 9          | Dog                   | 1.21  |   | 1.11                          | 1.03                         | 0.08                                     |   | 20             | 68 "                  |
|            |                       |   | 1.0   | 2.11                          | 0.75                         | 0.36                                     | 100                                       | 10             | 68 "                  |
| 10         | "                     | 0.93  |   | 0.90                          | 0.95                         | 0  |   | 20             | 53 "                  |
|            |                       |   | 1.0   | 1.90                          | 0.96                         | 0  | 94  | 10             | 53 "                  |
|            |                       |   | 2.0 (p.)  | 2.99                          | 0.69                         | 0.3                                      | 100                                       | 10             | 53 "                  |
| 11         | "                     | 0.86  |   | 1.00                          | 1.03                         | 0  |   | 15             | 65 "                  |
|            |                       |   | 1.0 (p.)  | 2.00                          | 1.17                         | 0  | 83  | 7.5            | 65 "                  |
|            |                       |   | 1.0   | 2.00                          | 0.80                         | 0.2                                      | 100                                       | 7.5            | 65 "                  |

TABLE I—*Concluded*

| Sample No. | Source of plasma used | Tungstomolybdate filtrate, Folin-Wu determination | Rubidium precipitation of saturated picric acid filtrates |                               |                              |  |   |                |
|------------|-----------------------|---|---|-------------------------------|------------------------------|--|---|----------------|
|            |                       |   | Creatinine added*   | Filtrate before precipitation | Filtrate after precipitation | Chromogenic plasma material precipitated | Per cent of added creatinine precipitated | Rubidium used† |
| 12         | Dog                   | 0.97  |   | 1.01                          | 1.15                         | 0  |   | 15             |
|            |                       |   | 1.0 (p.)  | 2.04                          | 1.05                         | 0  | 100                                       | 7.5            |
| 13         | "                     | 0.88  |   | 0.84                          | 0.86                         | 0  |   | 17             |
|            |                       |   | 1.0 (p.)  | 1.74                          | 0.75                         | 0  | 100                                       | 11             |
| 14         | Human (ultrafiltrate) | 0.88  |   | 0.80                          | 0.66                         | 0.14                                     |   | 10             |
| 15         | Human                 | 0.99  |   | 0.95                          | 1.02                         | 0  |   | 20             |
|            |                       |   | 1.0   | 1.95                          | 0.91                         | 0.04                                     | 100                                       | 10             |
| 16         | "                     | 0.94  |   | 0.94                          | 0.94                         | 0  |   | 10             |
|            |                       |   | 1.0   | 1.94                          | 0.70                         | 0.24                                     | 100                                       | 10             |
| 17         | "                     | 1.27  |   | 1.0                           | 0.88                         | 0.12                                     |   | 10             |
|            |                       |   |   | 1.0                           | 0.99                         | 0  |   | 20             |
|            |                       |   | 1.0   | 2.0                           | 0.80                         | 0.2                                      | 100                                       | 10             |
|            |                       |   | 1.0   | 2.0                           | 0.79                         | 0.2                                      | 100                                       | 20             |

\* Creatinine was added to the filtrate except where (p.) indicates that it was added to the plasma.

† The rubidium used was approximately the theoretical amount required to combine with the creatinine or chromogenic material present multiplied by the figure in this column.

same plasmas.\* Creatinine added to the plasma is quantitatively recovered.

Results obtained when the rubidium precipitation procedure was applied to fifteen filtrates of this sort from cat, sheep, beef, dog, and human plasma and from two ultrafiltrates from human and beef plasma are recorded in Table I. In most cases an iden-

\* The new type of plasma filtrate obtained in this way has presented an interesting problem which we hope to discuss in detail at a later time. The titratable acidity of these filtrates is always very considerably higher than that of pure saturated picric acid solutions, owing to the presence of additional picric acid. When the picric acid is completely extracted from these solutions with benzene, and the benzene evaporated, the weight of the dried residue is identical with the amount of picric acid calculated from the titration value. These findings are being further investigated.

tical procedure was applied to portions of each filtrate to which varying amounts of creatinine were added. In some cases the creatinine was added to the original plasma. In order to conserve space, similar results from several additional determinations with the same filtrates, with and without added creatinine, are not included in Table I. The amount of rubidium used is indi-

TABLE II

*Results of Rubidium Precipitation of Lloyd's-Treated, Picric Acid Plasma Filtrates to Which Creatinine Was Added*

Unless otherwise stated, figures are in terms of mg. of creatinine per 100 cc. of plasma.

| Plasma used<br>(figures refer to<br>Table I) | Before<br>Lloyd's<br>treat-<br>ment | After<br>Lloyd's<br>treat-<br>ment | Creati-<br>nine<br>added | Before<br>rubid-<br>ium<br>precipi-<br>tation | After<br>rubid-<br>ium<br>precipi-<br>tation | Per cent<br>of<br>added<br>creati-<br>nine<br>precipi-<br>tated | Rubid-<br>ium<br>used* | Time of<br>precipi-<br>tation |
|--|-------------------------------------|------------------------------------|--------------------------|---|--|---|------------------------|-------------------------------|
|  |                                     |                                    |                          |   |  |   |                        | hrs.                          |
| Beef (8)                                     | 2.10                                | 0.81                               |                          | 0.81  | 0.83   |   | 18                     | 72                            |
|  |                                     |                                    | 1.0                      | 1.81  | 0.85   | 96  | 8.3                    | 72                            |
| Dog (9)                                      | 1.11                                | 0.80                               |                          | 0.80  | 0.77   |   | 30                     | 72                            |
|  |                                     |                                    | 1.0                      | 1.80  | 0.77   | 100   | 14                     | 72                            |
| " (10)                                       | 0.90                                | 0.40                               | 0.5                      | 0.90  | 0.47   | 86  | 21                     | 49                            |
|  |                                     |                                    | 1.0                      | 1.40  | 0.44   | 96  | 13                     | 49                            |
|  |                                     |                                    | 2.0                      | 2.40  | 0.41   | 100   | 8                      | 49                            |
| " (11)                                       | 1.00                                | 0.47                               | 1.0                      | 1.47  | 0.48   | 100   | 10                     | 70                            |
|  |                                     |                                    | 2.0                      | 2.47  | 0.44   | 100   | 6.5                    | 70                            |
| " (12)                                       | 1.01                                | 0.49                               | 1.0                      | 1.49  | 0.47   | 100   | 14                     | 70                            |
|  |                                     |                                    | 2.0                      | 2.49  | 0.48   | 100   | 8                      | 70                            |
| " (13)                                       | 0.84                                | 0.43                               | 0.5                      | 0.93  | 0.47   | 92  | 20                     | 87                            |
|  |                                     |                                    | 1.0                      | 1.43  | 0.47   | 96  | 13                     | 87                            |
|  |                                     |                                    | 2.0                      | 2.43  | 0.47   | 98  | 10                     | 87                            |

\* The amount of rubidium used was approximately the theoretical amount required multiplied by the figures in this column.

cated in Table I and varied from 3 to 30 times the theoretical amount necessary, and the time of standing in the refrigerator varied from 24 hours to 5 days. Several of the picric acid filtrates were also treated with Lloyd's reagent to remove most of the chromogenic material,<sup>4</sup> creatinine was added to the Lloyd's-treated

<sup>4</sup> Removal of the chromogenic material with Lloyd's reagent from these filtrates was not complete. We have not investigated the conditions required for complete removal but think it possible that this might be effected by longer shaking with greater amounts of Lloyd's reagent.

filtrates thus obtained, and the same precipitation procedure applied. These results are recorded in Table II.

No appreciable amount of chromogenic material was precipitated from any of the plasma solutions to which creatinine had not been added (in only one case as much as 0.15 mg. per cent), although from pure creatinine solutions of corresponding concentrations all but 0.3 mg. per cent, or less, was precipitated by corresponding amounts of rubidium.

Added creatinine was usually entirely precipitated (over 90 per cent in all but two cases, when 75 and 83 per cent were precipitated).

In the presence of added creatinine a portion of the chromogenic material was occasionally also precipitated. This amounted to as much as 0.5 mg. per cent in one blood (beef, not included in Table I) and to 0.47, 0.36, and 0.3 mg. per cent in three others. The amount of chromogenic material removed in these cases was not comparable with the amount of creatinine precipitated from solutions of corresponding concentrations. In forty out of a total of forty-eight experiments the figures after precipitation agreed with those of the original filtrates to within less than 0.2 mg. per cent whether creatinine had been added or not, thus sharply differentiating the blood chromogenic material from creatinine.

Added creatinine was also removed practically quantitatively from Lloyd's-treated filtrates in thirteen out of a total of sixteen experiments. In two experiments with beef blood, not shown in Table II, 0.5 mg. per cent of creatinine was added to Lloyd's-treated plasma and practically none was precipitated, while 1 mg. per cent of creatinine added to one of the same filtrates was only 30 per cent precipitated. It may be noted that in the case of these bloods very poor plasma was obtained and the plasma filtrates which were used were not entirely free from protein, which we believe accounts for the failure to obtain precipitation of these small amounts of added creatinine.

According to Gaebler's report it is evidently possible, under suitable conditions, to precipitate a portion of the chromogenic material from dog serum ultrafiltrates. The technique which we employed failed to do this, although by the same procedure creatinine was precipitated from pure solution or after its addition to the filtrate. This finding presents another sharp difference

between the behavior of the creatinine chromogenic substance in blood and true creatinine.

#### SUMMARY

A technique, based on the findings of Greenwald and Gross and of Gaebler, is described, by means of which creatinine may be precipitated from very dilute solutions as the double creatinine rubidium picrate.

The new precipitation procedure was applied to plasma ultrafiltrates and to undiluted filtrates obtained by shaking plasma with solid picric acid. Plasma from various species was used.

The creatinine chromogenic material of plasma showed a marked contrast to creatinine (in pure solution or added to the filtrates) in that it was not appreciably precipitated by this procedure.

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## IMPROVEMENTS IN METHODS OF HYDROLYSIS OF PROTEIN: SHORTENING THE TIME FOR ESTIMATING CYSTINE\*

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In early studies on the biochemistry of sulfur, dealing primarily with the cystine content of proteins, Sullivan (1) sought to prevent the formation of both soluble and insoluble humin such as occurs when proteins are hydrolyzed with acid. It was thought that by prevention of the humin formation, loss of cystine in the insoluble humin and loss of cystine in the decolorization of the dark, soluble humin would be avoided. A procedure for hydrolyzing protein with little humin formation was early given by Hlasiwetz and Habermann (2) who used stannous chloride to check humin formation in the acid hydrolysis of casein. In work with casein, Sullivan found that stannous chloride would stop humin formation but it had the disadvantage that the tin was not only difficult to get rid of, but also that the procedure for eliminating tin was fraught with danger of loss of cystine. Accordingly, other means of preventing humin formation, by keeping a reducing atmosphere during hydrolysis, were tried and titanous chloride was found very satisfactory, since the titanium could be precipitated readily by neutralization with 5 N NaOH added dropwise, with stirring. The precipitation was complete at pH 6 or less. In the case of phaseolin, the filtered hydrolysate freed from titanium was reddish; the other filtrates were slightly yellow. In the hydrolysis of grass and brewers' yeast, however, humin formation was not stopped by titanium. As stated in previous publications (1, 3-5) the cystine found in casein, phaseolin, meat,

\* A preliminary report was presented at the Eighty-sixth meeting of the American Chemical Society at Chicago, September 14, 1933.



and fish in the titanium treatment was not sufficiently increased over that given by the ordinary hydrolysis with 20 per cent HCl to conclude that cystine was appreciably lost in humin formation despite the fact that the insoluble humin contained sulfur.

Later, considerable attention was given to hydrolyzing proteins in the presence of titanous chloride. When a 20 per cent titanous chloride solution, as bought but kept in the titanous stage by adding a little powdered zinc, was employed, it was found that the presence of titanous salts during the hydrolysis of protein not only tended to lessen or inhibit humin formation but also greatly lessened the time necessary for the complete liberation of the cystine.

Judging by the relation of amino nitrogen to total nitrogen, Levene and Bass (6) report that casein was completely hydrolyzed by 4 hours heating with 5 N HCl in sealed tubes. In our hydrolysis with 20 per cent HCl in an open system cystine is completely liberated only after 6 to 7 hours heating at 125°. As determined by the Sullivan method, which is positive only with free cystine, the hydrolysis is still incomplete after 2 or even 4 hours hydrolysis. The Okuda method, which gives positive values with complex  $\text{—SH}$  or  $\text{—S—S—}$  compounds, shows much higher values for cystine than the Sullivan method at the end of 2 hours hydrolysis. In both the Sullivan and the Okuda methods 1 to 2 hours hydrolysis in the presence of titanium give as good results as the 7 hours hydrolysis without titanium. The freshly prepared filtrate from the titanium contains only cysteine.<sup>1</sup> Accordingly, the cysteine content of the hydrolysate was determined by the Sullivan (7) cysteine procedure given briefly later in this paper with a slight modification. Then an aliquot was oxidized to cystine by a current of air and the cystine present determined against a cystine standard. In every case so far studied, both procedures gave results of the same order of magnitude.

*Hydrolysis of Protein in Presence of Titanous Chloride*—1 gm. of grain curd casein (8), 5 cc. of 20 per cent HCl, and 1 cc. of a

<sup>1</sup> In previous work with titanium reported by Sullivan (1) there was a long time interval (17 hours) between the end of hydrolysis and the estimation of cystine or cysteine. Under such conditions, cysteine and cystine were found in the titanium hydrolysate.

20 per cent solution of titanous chloride ( $\text{TiCl}_3$ ) were placed in a small acetylation flask fitted with a ground glass reflux condenser. The mixture was heated in an oil bath at  $125^\circ$  for 1 to 2 hours. Then the mixture was poured into a small beaker and the flask washed with 5 cc. of water. The total solution was then neutralized with 5 N NaOH, added dropwise with stirring, to about pH 6. The titanium was precipitated mainly as  $\text{TiO}_2\cdot\text{H}_2\text{O}$ . The solution was filtered by suction from the blue, somewhat gelatinous titanous hydroxide, and the precipitate was washed with 5 cc. of water. The total filtrate adjusted to pH 3.5 with concentrated HCl was made to 35 cc. with 0.1 N HCl. The solution gave a direct nitroprusside reaction when 1 cc. was tested with 0.3 cc. of a 4 per cent aqueous solution of sodium nitroprusside and 0.5 cc. of concentrated  $\text{NH}_4\text{OH}$  (28 per cent). As a rule the further addition of NaCN did not increase the color given by the fresh filtrate from the titanium treatment. Of this filtrate 5 cc. were taken for the determination of cysteine<sup>2</sup> by the Sullivan procedure and 10 cc. for the Okuda (9) cysteine procedure. Through the rest of the solution air was bubbled until the direct test for  $-\text{SH}$  compounds by sodium nitroprusside was negative.

*Cysteine by Sullivan Method*—To 5 cc. of the hydrolysate add 1 cc. of a 0.5 per cent aqueous solution of 1,2-naphthoquinone-4-sodium sulfonate; shake 10 seconds and add 5 cc. of 10 per cent anhydrous  $\text{Na}_2\text{SO}_3$  in 0.5 N NaOH, then 1 cc. of 1 per cent NaCN in 0.8 N NaOH; mix and wait 30 minutes. Then add 2 cc. of 5 N NaOH and 1 cc. of a 2 per cent solution of sodium hyposulfite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) in 0.5 N NaOH. Match against 5 cc. of a cysteine standard containing 0.5 to 1 mg. of cysteine (0.65 to 1.3 mg. of cysteine hydrochloride) similarly treated.

*Cysteine by Okuda Method*—To 10 cc. of filtrate add 0.5 cc. of concentrated HCl, 2.5 cc. of 5 per cent KI, and 2.5 cc. of 4 per cent HCl. Bring to  $20^\circ$  and titrate with  $\text{m}/600$   $\text{KIO}_3$  previously standardized against cysteine.

In the solution oxidized by air the cystine procedure was used in both the Sullivan and the Okuda methods as follows:

*Cystine by Sullivan Method*—To 5 cc. of solution add 2 cc. of

<sup>2</sup> In the titanium procedure described above free cystine was found to be converted to cysteine quantitatively.

aqueous 5 per cent NaCN, or better 2 cc. of 5 per cent NaCN in *N* NaOH; mix well and wait 10 minutes, with the temperature of the solution not lower than 20°. Then add the naphthoquinone, the sodium sulfite, etc., as in the cysteine procedure

TABLE I

*Cystine Content of Several Proteins,\* Hydrolyzed without Titanium (A) and Hydrolyzed in Presence of TiCl<sub>3</sub> (B)*

| Protein            | Time of hydrolysis | Treatment       | Cystine found   |              |                      | Estimated as |
|--------------------|--------------------|-----------------|-----------------|--------------|----------------------|--------------|
|                    |                    |                 | Sullivan method | Okuda method | Folin-Maclean method |              |
|                    | hrs.               |                 | per cent        | per cent     | per cent             |              |
| Casein (A)         | 7                  | Decolorized     | 0.304           | 0.304        |                      | Cystine      |
| " "                | 2                  | "               | 0.140           | 0.280        |                      | "            |
| " (B)              | 2                  | {               | 0.315           | 0.304        |                      | Cystine      |
| " "                | 1                  |                 | 0.304           | 0.304        |                      | Cystine      |
| " "                | 1                  |                 | 0.315           | 0.304        |                      | Cystine      |
| Lactalbumin (A)    | 8                  | Decolorized     | 2.29            | 2.44         | 3.98                 | Cystine      |
| " "                | 2                  | "               | 1.36            | 1.95         |                      | "            |
| " (B)              | 2                  | {               | 2.38            | 2.51         |                      | Cystine      |
| " "                | 1                  |                 | 2.30            | 2.47         |                      | Cystine      |
| " "                | 1                  |                 | 2.13            | 2.14         |                      | Cystine      |
| Serum globulin (A) | 7                  | {               | 1.82            | 1.92         | 2.25                 | Cystine      |
| " " "              | 2                  |                 | 1.74            | 1.80         |                      | "            |
| " " (B)            | 7                  |                 | 1.88            | 1.96         |                      | Cystine      |
| " " "              | 2                  |                 | 1.87            | 1.96         |                      | "            |
| " "                | 2                  |                 | 1.87            | 1.96         |                      | "            |
| Zein (A)           | 7                  | Decolorized     | 0.76            | 0.80         | 0.84                 | Cystine      |
| " "                | 7                  | Not decolorized | 0.86            | 0.88         | 0.96                 | "            |
| " "                | 2                  | " "             | 0.57            | 0.88         |                      | "            |
| " (B)              | 1                  |                 | 0.89            | 0.90         |                      | Cystine      |

\* With the exception of the serum globulin the cystine and cysteine values are corrected for moisture and ash.

but omit the 1 per cent NaCN. Match against 5 cc. of a suitable cystine standard similarly treated with NaCN, etc.

*Cystine by Okuda Method*—To 10 cc. of the solution add 0.5 cc. of concentrated HCl and a small amount of powdered zinc, gently warm for a few minutes, and let stand for 1 hour at room

temperature to reduce the cystine; filter and wash. Adjust the reaction to 2 per cent HCl and proceed as in the Okuda cysteine method.

With lactalbumin, serum globulin, and zein the amounts used for hydrolysis were 0.5 gm., 0.25 gm., and 0.25 gm. respectively. Otherwise the procedure was as with casein. The lactalbumin was obtained from Dr. D. B. Jones, the serum globulin from Dr. A. E. Mirsky, and the zein from Dr. H. B. Vickery.

In the titanium procedure given in this paper, difficulty was met with only in the case of casein. For satisfactory results 1 gm. or, better, 2 gm. of casein must be hydrolyzed. Occasionally also a high yellow color appeared in the casein filtrate from the titanium hydrolysate and interfered with the colorimetric reading. The data given as to casein in this paper are from determinations in which no difficulty was met with.

The results with the various proteins by the ordinary HCl hydrolysis for 2 and 7 hours and by the short hydrolysis in the presence of titanium are given in Table I. Because of lack of material the 1 hour hydrolysis with titanium was not carried out with the serum globulin.

The high values given for lactalbumin and serum globulin by the Folin-Marenzi method (10) are due to substances other than cystine. Cystine treated with sodium cyanide will not give a blue color with the Folin-Marenzi reagent. The hydrolysates of the two proteins mentioned above still give a blue in the presence of sodium cyanide.

The serum globulin in the ordinary HCl hydrolysis was brownish, but this color was discharged by the sodium hyposulfite and did not interfere in the colorimetric estimation.

With zein one 7 hour hydrolysate was decolorized by use of 50 mg. of carboraffin.<sup>3</sup> In another tan-colored hydrolysate it was found that the color was discharged by reducing agents such as were used in the cystine determination, so the hydrolysate was used without decolorizing.

#### SUMMARY

By hydrolyzing protein in the presence of titanous chloride, humin formation is lessened and the time required for hydrolysis

<sup>3</sup> Carboraffin is now known as carbex E.

of the protein to liberate cystine as cysteine is greatly shortened. The results are of the same order of magnitude whether cysteine is determined directly or as cystine after oxidation by air.

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# THE PROTEOLYTIC ENZYMES OF SOME COMMON MOLDS\*

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Although considerable work has been done on the proteolytic activity of molds, very little of it is concerned with the individual components of the proteolytic system. In previous papers (1, 2), it has been shown that the proteolytic system of *Aspergillus parasiticus* contains a proteinase, a dipeptidase, an aminopolypeptidase, a carboxypolypeptidase, and enzymes that hydrolyze diglycine and triglycine. It was therefore considered worth while to investigate a number of species in order to determine the similarity or non-similarity of their proteolytic systems to that of *Aspergillus parasiticus* and to determine the extent of variation existing among the different species studied.

## EXPERIMENTAL

### Methods

About thirty species were grown on two media, a 10 per cent glucose-inorganic salts medium and a 5 per cent glucose-skim milk medium; the composition of these has already been described in detail (3). 100 cc. portions of medium were placed in 500 cc. Erlenmeyer flasks and after autoclaving were inoculated with 0.5 cc. of spore suspension. Each mold was grown on each medium in duplicate flasks. The flasks were incubated at 28-30°, and the mycelium was harvested at the time of sporulation, or, in about 10 days if sporulation had not occurred before that time; some slow growing species, however, were incubated for longer periods. Previous work had shown that the maximum

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enzyme content of the mycelium was attained at the time of sporulation. The mycelial mats were washed with water and squeezed out by hand. A 1.0 gm. sample was dried at 105° for 6 hours for determination of dry matter.

In order to ascertain a convenient and efficient way of extracting the enzymes, a number of methods were tried. These included extraction with water for various lengths of time and at different pH values, extraction by grinding with sand, and extraction with NaCl solution. The following method was adopted. The mycelium was ground very thoroughly in a mortar, and diluted so that the total amount of water was 10 times as much as the calculated amount of dry mold. The pH was adjusted to 7.0 colorimetrically, a little toluene was added, and the mixture was allowed to stand at room temperature for 6 hours. It was then centrifuged and the supernatant liquid was analyzed for its enzyme content. In all preliminary determinations, 1.0 cc. of this solution, representing 0.1 gm. of dry mycelium, was used. Dilution to the proper concentration, if necessary, could then be made for more accurate determinations.

The proteinase, carboxypolypeptidase, aminopolypeptidase, and dipeptidase contents and the diglycine- and triglycine-splitting activities of the extract were determined by the extent of hydrolysis of gelatin, chloroacetyl-*l*-tyrosine, *dl*-leucyldiglycine, *dl*-leucylglycine, diglycine, and triglycine respectively. The methods of enzyme determination and the units for expressing enzyme activity have been previously described (1, 2). They were followed except for certain slight modifications. On account of the difficulty involved in the preparation of the peptidase substrates, the amount of solution used per determination was reduced to half that described in (2). Thus 2.0 cc. of stock substrate solution were diluted to 3.0 cc. with enzyme and water, and 1.0 cc. aliquots of the mixture were titrated. Since diglycine and triglycine substrates had not been previously used, their composition is given here. 0.005 M diglycine was dissolved in water, 5.0 cc. of N NaOH added, and the solution diluted to 100 cc. The pH of this solution was about 8.0. A solution of 0.05 M triglycine was made in a similar way.

*Kinetics of Enzymes*—To justify the use of the units employed, the kinetics of the proteinase, aminopolypeptidase, carboxypoly-

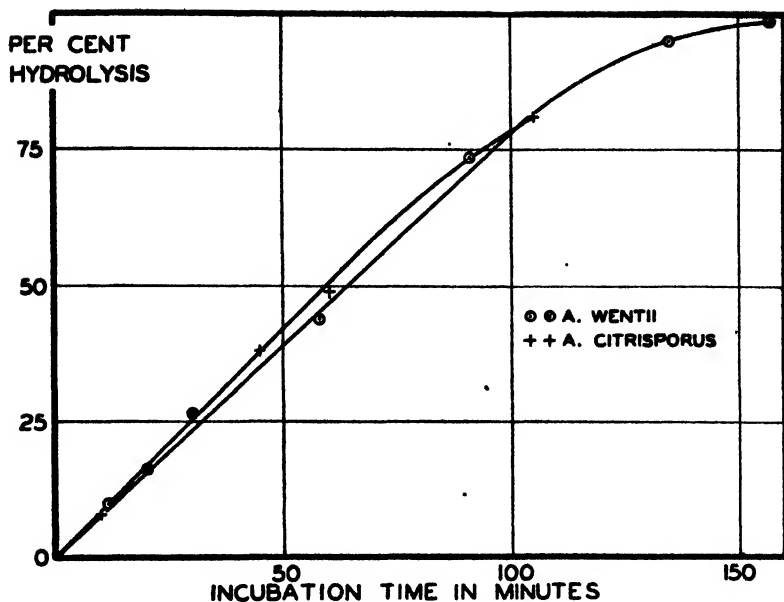


FIG. 1. Kinetics of dipeptidase activity

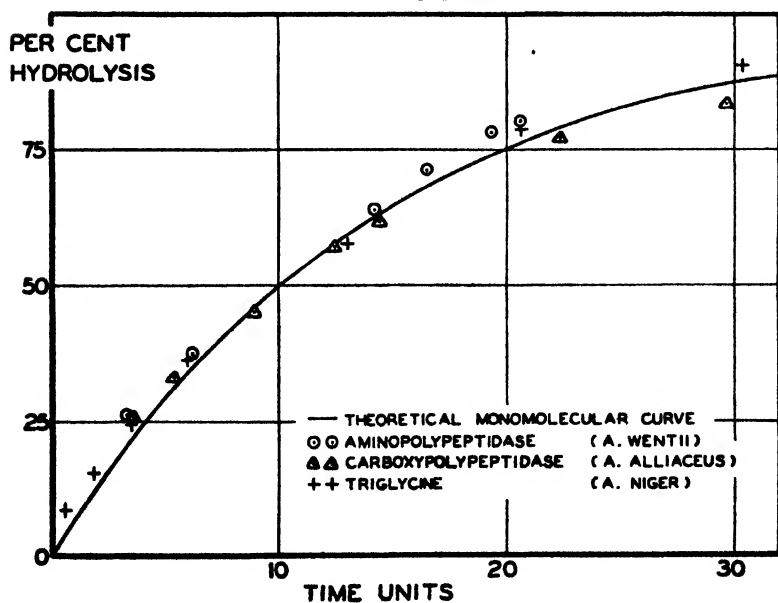


FIG. 2. Kinetics of the polypeptidases. This figure has been set up so that half hydrolysis of any substrate occurs in 10 time units.



peptidase, and dipeptidase action were studied on enzyme solutions from the following molds: *Aspergillus alliaceus*, *A. citriporus*, *A. wentii*, *Penicillium citrinum*, *P. terrestre*, and *A. melleus*. Fig. 1 shows the type of reaction curve obtained with two of the molds for dipeptidase. It can be seen that the hydrolysis of leucylglycine was a zero order reaction. The proteinase showed a similar linear curve up to 2 cc. of gas liberated, and hence the determinations were arranged so that 2 cc. or less of gas were always formed. Fig. 2 illustrates the kinetics of the aminopolypeptidase, the carboxypolypeptidase, and the triglycine-splitting enzymes. The proximity of the experimental points to the theo-

TABLE I  
*Enzyme Content of Aspergillus alliaceus*

| Enzyme                       | Volume of extract | Weight of dry mycelium | Gas or titration | Relation between degree of hydrolysis and amount of enzyme | Units in sample | Units in 200 gm. dry mold |
|------------------------------|-------------------|------------------------|------------------|--|-----------------|---------------------------|
|                              | cc.               | gm.                    | cc.              |  |                 |                           |
| Proteinase . . . .           | 0.1               | 0.01                   | 1.41             | 2.24 cc. = 0.005 unit                                      | 0.00315         | 63.0                      |
| Carboxypolypeptidase . . . . | 0.5               | 0.05                   | 0.285            | $E = \frac{1}{2t} \log \frac{a}{a-x}$                      | 0.00306         | 12.2                      |
| Aminopolypeptidase . . . .   | 0.2               | 0.02                   | 0.205            | Same   | 0.0382          | 38.2                      |
| Dipeptidase . . . .          | 0.2               | 0.02                   | 0.220            | $E = 0.602 x/t$  | 0.0022          | 22.0                      |

$E$  = number of enzyme units present in reaction mixture;  $t$  = incubation time in minutes, in this case 60 minutes;  $a$  = 0.50, the titration corresponding to total hydrolysis;  $x$  = observed titration.

retical monomolecular curve justifies the use of the formula for first order reactions in the calculations of the enzyme units for the hydrolysis of leucyldiglycine, chloroacetyl-*L*-tyrosine, and triglycine. Diglycine enzyme units were also calculated from the monomolecular formula on the basis of previous work done with *Aspergillus parasiticus*.

*Variation of Enzyme Content with Species and Medium*—Space does not permit giving the detailed experimental observations for all the cultures; hence, to illustrate the relations between the observed readings and the units reported, Table I has been set up for one species, *Aspergillus alliaceus*. Calculations for the other species were made in a similar manner.

TABLE II  
Enzyme Contents of Mold Mycelium

| Mold                         | Medium | Incubation period | Yield of mycelium | Enzyme units per 200 gm. of dry mold tissue |             |                     |                       |                  |                   |
|------------------------------|--------|-------------------|-------------------|---|-------------|---------------------|-----------------------|------------------|-------------------|
|                              |        |                   |                   | Proteinase                                  | Dipeptidase | Aminopoly-peptidase | Carboxypoly-peptidase | Diglycine enzyme | Triglycine enzyme |
|                              |        | days              | gm. per 200 cc.   |   |             |                     |                       |                  |                   |
| <i>A. alliaceus</i>          | Salts  | 7                 | 5.9               |   | 3.2         | 4.3                 | 1.5                   |                  |                   |
|                              | Milk   | 12                | 6.1               | 63.0  | 22.0        | 38.2                | 12.2                  |                  |                   |
| " <i>citrisporus</i>         | Salts  | 4                 | 5.7               | 2.6   | 13.4        | 21.3                | 9.7                   | 1.4              | 4.0               |
|                              | Milk   | 10                | 6.2               | 4.0   | 58.0        | 16.0                | 7.4                   | 2.7              | 3.4               |
| " <i>fischeri</i> , single   | Salts  | 4                 | 2.5               | 6.0   | 7.4         | 16.5                | 5.6                   |                  |                   |
| spore culture No. 20         | Milk   | 12                | 3.2               | 5.2   | 11.4        | 20.1                | 5.1                   |                  |                   |
| " <i>melleus</i>             | Salts  | 11                | 4.3               |   | 4.2         | 0.9                 | 0.0                   | 0.7              | 0.3               |
|                              | Milk   | 11                | 4.4               |   | 6.0         | 8.1                 | 32.0                  | 1.8              | 2.9               |
| " <i>niger</i> , No. 2       | Salts  | 4                 | 6.9               | 2.0   | 6.8         | 5.6                 | 8.5                   | 2.9              | 10.0              |
|                              | Milk   | 4                 | 1.8               | 1.4   | 5.6         | 2.3                 | 5.1                   |                  |                   |
| " <i>oryzae</i> , No. 1      | Salts  | 30                | 2.4               |   | 3.4         | 0.2                 | 0.2                   |                  |                   |
|                              | Milk   | 12                | 2.7               | 0.6   | 186         | 45.0                | 5.1                   |                  |                   |
| " " " 965                    | Salts  | 10                | 1.8               | 3.6   | 40.0        | 62.8                | 4.8                   |                  |                   |
|                              | Milk   | 13                | 4.1               | 10.4  | 141         | 50.0                | 6.9                   |                  |                   |
| " <i>parasiticus</i>         | Salts  | 5                 | 4.0               | 3.2   | 8.6         | 36.8                | 7.6                   | 0.6              | 1.2               |
|                              | Milk   | 6                 | 3.6               | 11.4  | 41.0        | 24.0                | 18.0                  | 2.3              | 5.6               |
| " <i>sydowi</i>              | Salts  | 9                 | 5.2               | 1.8   | 1.2         | 17.1                | 2.3                   |                  |                   |
|                              | Milk   | 24                | 4.2               | 0.8   | 0.0         | 15.2                | 0.5                   |                  |                   |
| " <i>terreus</i>             | Salts  | 7                 | 7.7               | 2.6   | 6.6         | 6.3                 | 1.1                   | 0.0              | 0.6               |
|                              | Milk   | 9                 | 7.0               | 9.8   | 12.6        | 8.1                 | 1.1                   | 0.1              | 1.8               |
| " <i>wentii</i> , No. 44,173 | Salts  | 4                 | 4.8               | 0.0   | 8.6         | 21.3                | 4.2                   |                  |                   |
|                              | Milk   | 7                 | 5.5               | 12.2  | 61.0        | 32.2                | 8.1                   |                  |                   |
|                              | Salts  | 4                 | 3.2               | 2.0   | 2.6         | 20.7                | 5.1                   |                  |                   |
|                              | Milk   | 6                 | 6.0               | 8.2   | 100         | 22.3                | 10.3                  |                  |                   |
| <i>Cunninghamella</i>        | Salts  | 21                | 6.1               | Trace                                       | 1.8         | 6.6                 | 0.7                   |                  |                   |
|                              | Milk   | 21                | 3.0               | "   | 7.0         | 11.5                | 1.5                   |                  |                   |
| <i>P. camemberti</i> , No. 2 | Salts  | 42                | 4.8               | 0.4   | 4.0         | 5.1                 | 0.6                   | 0.4              | 0.4               |
|                              | Milk   | 42                | 5.2               | 1.6   | 2.8         | 25.0                | 6.2                   | 1.4              | 1.5               |
| " <i>caseicola</i>           | Salts  | 26                | 6.0               | 1.8   | 1.6         | 80                  | 6.0                   |                  |                   |
|                              | Milk   | 36                | 6.1               | Trace                                       | 1.0         | >40                 | 3.8                   |                  |                   |
| " <i>chrysogenum</i>         | Salts  | 15                | 5.8               | 0.4   | 2.0         | 18.4                | 8.7                   |                  |                   |
|                              | Milk   | 12                | 3.5               | 2.4   | 2.8         | 16.5                | 1.4                   |                  |                   |
| " <i>citrinum</i>            | Salts  | 5                 | 2.9               |   | 0.9         | 37.0                | 2.1                   |                  |                   |
|                              | Milk   | 20                | 4.3               | 1.8   | 0.2         | 78.1                | 2.2                   |                  |                   |

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TABLE II—Concluded

| Mold                         | Medium | Incubation period | Yield of mycelium | Enzyme units per 200 gm. of dry mold tissue |             |                     |                       |                  |                   |
|------------------------------|--------|-------------------|-------------------|---|-------------|---------------------|-----------------------|------------------|-------------------|
|                              |        |                   |                   | Proteinase                                  | Dipeptidase | Aminopoly-peptidase | Carboxypoly-peptidase | Diglycine enzyme | Triglycine enzyme |
|                              |        | days              | gm. per 100 cc.   |   |             |                     |                       |                  |                   |
| <i>P. janthinellum</i>       | Salts  | 15                | 7.9               | 1.0   | 1.8         | 9.7                 | 0.7                   |                  |                   |
|                              | Milk   | 12                | 5.5               | 3.0   | 3.0         | 3.4                 | 0.2                   |                  |                   |
| " <i>terrestre</i>           | Salts  | 10                | 4.2               | 5.8   | 2.8         | 66.3                | 5.6                   |                  |                   |
|                              | "      | 5                 | 4.5               |   | 1.3         | 48.0                | 2.6                   |                  |                   |
|                              | Milk   | 28                | 5.8               |   | 0.6         | 126                 | 4.5                   | 0.9              | 1.7               |
|                              | "      | 9                 | 3.5               |   | 0.7         | 178                 | 3.1                   |                  |                   |
| <i>Coprinus radians</i>      | "      | 44                | 4.3               |   | 14.5        | 35.0                | 0.8                   |                  |                   |
| <i>Agaricus campestris</i> * |        |                   |                   | 3.4   | 3.6         | 5.0                 | 13.3                  |                  |                   |

\* Fresh whole mushroom was ground and extracted.

Table II contains a summary of the enzyme contents of nineteen species of molds. It should be pointed out that tabulated 0 values for enzyme content do not mean the entire absence of those enzymes but merely indicate that no hydrolysis of the substrates was observed in 1 hour.

The remaining species, which are not tabulated, could be classified roughly as follows: (1) contents of aminopolypeptidase, carboxypolypeptidase, and dipeptidase less than 10 units per 200 gm. of dry mycelium, *Hormodendrum* sp., *Penicillium aurantio-brunneum*, *P. italicum*, and *P. lilacinum*; (2) dipeptidase and carboxypolypeptidase contents less than 10 units but aminopolypeptidase content 10 to 30 units, *Aspergillus carbonarius*, *A. nidulans*, *A. fischeri*, single spore culture No. 7, *Penicillium chloro-leucon*, *P. dierckxii*, *P. restrictum*, and *P. varioti*.

In one series of experiments the amounts of the constituents of the two media were varied to see what the effect would be on the enzyme content of the mold pad. Although variations occurred, these were not generally so large as to be especially notable; with one-tenth the usual glucose concentration in the

milk medium, the pad yield was considerably smaller, while the enzyme content was the maximum per gm. of dry weight.

The validity of the view that the cleavage of the six substrates is the work of six different enzymes is supported by previous fractionation experiments and the activity ratios of the different enzymes. Johnson and Peterson (2) suggested that the enzyme of *Aspergillus parasiticus* that split leucylglycine was probably not identical with the one that split diglycine; similarly, triglycine was not split by a purified solution of aminopolypeptidase. An inspection of the data obtained in this study brings out clearly the same facts. Thus if it were the same enzyme in *Penicillium terrestre* that split leucyldiglycine and leucylglycine in the ratio of 254:1, it is to be expected that this enzyme would maintain the same ratio in *Aspergillus wentii*. But this is definitely not so, the ratio being 0.22:1. Therefore it is indicated that two enzymes are involved in these hydrolyses. Table III contains a summary of data which illustrate the above relations for other paired substrates. The higher the value in the last column, the more indicative the data are that two different enzymes are involved in the splitting of the paired substrates. The possibility that accompanying substances influence the actions of one enzyme on different substrates should be kept in mind; other work from this laboratory, however, tends to substantiate the existence of the number of different enzymes reported in this paper.

From the data of Tables II and III, the following conclusions may be drawn.

1. All the molds analyzed contained at least four proteolytic components; in addition, two more components that split diglycine and triglycine were found in most of the molds which were analyzed for these enzymes. They apparently occur in considerably smaller amounts than do the other peptidases.

2. The molds contain the different components in widely varying amounts, no one mold being highest in all the components. The peptidases seemed to vary much more than the proteinase.

3. The enzyme content varied as much between members of the same genus as between molds of different genera. In general, the *Aspergilli* contained more proteolytic enzyme than the *Penicillia*. A similar relationship has been reported for amylase (4).

4. The medium upon which the mold has been grown and the

## 436 Proteolytic Enzymes of Common Molds

period of incubation have an important effect on the amount of enzyme formed. Molds grown on an organic medium usually contained more enzyme than those grown on a synthetic medium.

TABLE III

*Activity Ratios Toward Pairs of Substrates (Crude Mold Enzymes)*

The following abbreviations have been used: lg = leucylglycine; lgg = leucyldiglycine; ggg = triglycine; gg = diglycine; Clat = chloroacetyl-*l*-tyrosine.

| Ratio of substrates | Highest ratio |                           | Lowest ratio |                                 | Ratio Highest Lowest |
|---------------------|---------------|---------------------------|--------------|---------------------------------|----------------------|
| lgg:lg              | 254           | ( <i>P. terrestre</i> )   | 0.22         | ( <i>A. wentii</i> )            | 1140                 |
| lgg:ggg             | 74            | " "                       | 0.56         | (" <i>niger</i> )               | 132                  |
| lgg:gg              | 140           | " "                       | 1.9          | " "                             | 74                   |
| lgg:Clat            | 57            | " "                       | 0.25         | (" <i>melleus</i> )             | 230                  |
| lg:ggg              | 17            | ( <i>A. citrisporus</i> ) | 0.61         | ( <i>P. aurantio-brunneum</i> ) | 25                   |
| lg:gg               | 22            | " "                       | 0.67         | ( <i>P. terrestre</i> )         | 33                   |
| lg:Clat             | 36            | (" <i>oryzae</i> , No. 1) | 0.19         | ( <i>A. melleus</i> )           | 190                  |
| ggg:gg              | 3.4           | (" <i>niger</i> )         | 1.0          | ( <i>P. camemberti</i> )        | 3.4                  |
| ggg:Clat            | 3.0           | (" <i>parasiticus</i> )   | 0.1          | ( <i>A. melleus</i> )           | 30                   |
| gg:Clat             | 1.5           | " "                       | 0.056        | " "                             | 27                   |

TABLE IV

*Optimum pH for Proteinase of Aspergillus oryzae, No. 965*

| pH | Increase in gas | pH | Increase in gas |
|----|-----------------|----|-----------------|
|    | cc.             |    | cc.             |
| 9  | 1.16            | 5  | 0.96            |
| 8  | 1.46            | 4  | 0.25            |
| 7  | 1.71            | 3  | 0.05            |
| 6  | 1.25            |    |                 |

To 9.0 cc. of gelatin at the different hydrogen ion concentrations was added 1.0 cc. of crude enzyme solution, plus the amount of acid or alkali necessary to bring this 1.0 cc. of extract to the same pH, as predetermined. Incubation was for 1 hour at 40°.

This was especially true of the *Aspergilli*, while in the *Penicillia* only the aminopolypeptidase production was stimulated by organic N.

5. Molds that grew especially well on either medium tended to have only a small amount of enzyme per unit of dry weight.

6. While different batches of the same molds occasionally gave widely varying enzyme content, on the whole the enzyme yields were fairly reproducible.

*Optimum pH for Proteinase*—Various workers (5-7) have reported a wide range of pH optima for mold proteinase, depending both on the substrate and the organism used. With enzyme solutions from *Aspergillus oryzae*, No. 965, *A. alliaceus*, and *A. wentii* (grown on milk medium), the optimum pH for the hydrolysis of gelatin was found to be about pH 7. The data obtained with one of these molds are included in Table IV.

#### SUMMARY

1. The proteolytic enzyme content of thirty common molds has been studied in detail. The proteolytic system consists of at least one proteinase and at least five peptidases; namely, a dipeptidase, a carboxypolypeptidase, an aminopolypeptidase, and two enzymes that hydrolyze diglycine and triglycine.

2. The kinetics of the liberation of amino nitrogen from gelatin and of the hydrolysis of leucylglycine, leucyldiglycine, chloroacetyl-*L*-tyrosine, and triglycine by mold enzymes have been studied. While the hydrolysis of gelatin and leucylglycine was a zero order reaction, the hydrolysis of the other peptides was a first order reaction.

3. The molds contain the different components in widely varying amounts. The *Aspergilli* are generally higher in enzyme content than the *Penicillia*, but there is a great variation between individual members of the same genus.

4. The amounts of the components vary with the medium upon which the mold has been grown and with the period of incubation.

5. The optimum hydrogen ion concentration for the proteinase of three molds acting on gelatin was found to be about pH 7.

The organisms used in this study are the same as those that have been used for the past several years in this laboratory for chemical investigation. The cultures were supplied and their purity checked by Dr. H. C. Greene, to whom the authors wish to express their sincere thanks.

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## THE CARBAMATE EQUILIBRIUM\*

### II. THE EQUILIBRIUM OF OXYHEMOGLOBIN AND REDUCED HEMOGLOBIN

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For a considerable number of years the idea has persisted that a form of  $\text{CO}_2$  other than bicarbonate and free  $\text{CO}_2$  exists in protein solutions, particularly hemoglobin. The hypothesis which states this moiety of  $\text{CO}_2$  to be a protein carbamate, that is, an ionized protein "salt" ( $\text{Prot}\cdot\text{NH}\cdot\text{COO}^-$ ) of protein carbamic acid ( $\text{Prot}\cdot\text{NH}\cdot\text{COOH}$ ), has lately gained considerable experimental support. The original observations of Bohr (1905) on "carbo-hemoglobin," a supposed direct combination of  $\text{CO}_2$  and hemoglobin, the work of Siegfried (1905) on carbamates in serum, the researches of Faurholt (1925) on carbamates in solutions of amino acids, the observations of Henriques (1928) on the kinetics of  $\text{CO}_2$  uptake by hemoglobin solutions, the contributions of Roughton and his coworkers on the theory of the carbamino compounds and on the methods pertaining thereto, and, finally, the data of this paper, lead to the conclusion that the carbamate form of  $\text{CO}_2$  exists in all protein solutions in equilibrium with  $\text{CO}_2$ . In the case of the blood, it plays an important rôle in  $\text{CO}_2$  transport.

A necessary further development of the subject was the application of the mass law to the carbamate equilibrium in hemoglobin solutions. In Paper I of this series (Stadie and O'Brien, 1935-36) it was shown on the basis of simple assumptions that the carbamate equilibrium of simple amino acids can be described by the mass action law. In this paper we believe we have shown on the basis

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of similar assumptions that the mass law also can describe adequately the carbamate equilibrium in the case of hemoglobin and serum proteins. From the equations developed, it is possible to calculate equilibrium constants for the hemoglobin-carbamate equilibrium, and to calculate the carbamate concentration in hemoglobin solutions over a wide range of conditions. The evaluation of the rôle of carbamate as a carrier of  $\text{CO}_2$  in the red cells and serum depends upon such a quantitative understanding of the equilibrium.

*Nature of Combination of  $\text{CO}_2$  and Hemoglobin*—Bohr (1905) believed he had demonstrated the existence of a direct combination of  $\text{CO}_2$  and hemoglobin. According to him, this combination was one involving only that group of the hemoglobin molecule which also binds oxygen. Faurholt (1925) conclusively demonstrated a direct combination of  $\text{CO}_2$  with the  $-\text{NH}_2$  group of simple amino acids. Henriques (1929) proposed that similar direct combination of  $\text{CO}_2$  and  $-\text{NH}_2$  occurs in the case of the more complex amino acid, hemoglobin. But Henriques, and also Margaria and Green (1933), found that the maximum amount of  $\text{CO}_2$  which combined directly with hemoglobin as carbamate was equal to the oxygen capacity. Accordingly, and in conformity with Bohr's idea, they asserted that the  $\text{CO}_2$  combined with a single amino group of the oxygen linkage exclusively and not with any other. On the contrary we find (see Table II) that the demonstrable amount of  $\text{CO}_2$  combined as carbamate may under suitable conditions be 3 or 4 times the oxygen capacity. It may be concluded, therefore, that the carbamate combination is not limited exclusively to the oxygen-binding group, but involves many if not all of the  $-\text{NH}_2$  groups of the hemoglobin molecule.

It will be shown, in confirmation of the early work of Siegfried (1905), that carbamate formation is not peculiar to hemoglobin, but can be demonstrated in the case of horse serum protein. This fact is additional evidence that this combination is not limited to a prosthetic "gas"-combining group.

Faurholt (1925) further proposed, in the case of the simple amino acids, the hypothesis that the direct combination of  $\text{CO}_2$  is with the amphanion ( $\text{COO}^-\cdot\text{R}\cdot\text{NH}_2$ ) only and not with the zwitter ion ( $\text{COO}^-\cdot\text{R}\cdot\text{NH}_3^+$ ); and Meldrum and Roughton (1933) and Stadie and O'Brien (1935-36) have discussed the experimental

evidence which substantiates this hypothesis. Analogous evidence that this hypothesis applies in the case of hemoglobin, namely that  $\text{CO}_2$  combines only with the protein amphanion (i.e. hemoglobinate,  $\text{Hb}^-$ ) and not with the protein zwitter ion (i.e. isoelectric hemoglobin), is summarized here.

1. There is evidence in the literature showing that the ionization of hemoglobin is similar to that of simple amino acids. At the isoelectric pH ( $=\text{pI}$ ), only zwitter ions ( $\text{COO}^- \cdot \text{Hb} \cdot \text{NH}_3^+$ ) exist. On the alkaline side of  $\text{pI}$ , these ionize into amphanions ( $\text{COO}^- \cdot \text{Hb} \cdot \text{NH}_2$  or  $\text{Hb}^-$ ) which increase proportionately to the increase of pH according to the equation

$$(\text{Hb}^-) = \beta_{\text{Hb}}(\text{Hb}) (\text{pH} - \text{pI}) \quad (1)$$

Here  $(\text{Hb}^-)$  is the concentration in equivalents of the amphanion,  $(\text{Hb})$  is the total molar concentration of hemoglobin (oxygen capacity), and  $\beta_{\text{Hb}}$  is the "buffer value." Equation 1, the buffer equation of hemoglobin, has been shown by the work of Van Slyke and his coworkers (1922) to hold over a considerable range of pH.

2. According to the carbamate hypothesis, hemoglobin at isoelectric pH (or less), at which pH it contains no amphanion, should not form carbamate when brought to either "non-carbonate" or "total" equilibrium with  $\text{CO}_2$ .<sup>1</sup> Experimentally it is shown, in Table I, that this is so. In the case of the non-carbonate equilibrium, an initially isoelectric hemoglobin solution when brought to equilibrium with  $\text{CO}_2$  had a ratio of carbamate to hemoglobin,  $(\text{HbAm}^-)/(\text{Hb})$ , of 0 at isoelectric pH; even  $\text{CO}_2$  at markedly increased partial pressures failed to combine with the hemoglobin as carbamate. In the case of the total equilibrium likewise, an initially alkaline solution of either oxyhemoglobin or reduced hemoglobin at  $38^\circ$  showed 0 carbamate at isoelectric pH when brought to this pH by sufficient increase of the equilibrium  $P_{\text{CO}_2}$ . (The carbamate was determined directly by the Ferguson-Roughton (1934) method.)

3. Since a hemoglobin solution made alkaline by the addition of available base ( $\text{B}^+$ ) contains amphanion ( $\text{Hb}^-$ ) initially equal to the base, and since the carbamate in a hemoglobin solution in

<sup>1</sup> The distinction between the "non-carbonate" and the "total" equilibrium is discussed later.

non-carbonate equilibrium with  $\text{CO}_2$  approaches a maximum as the equilibrium  $P_{\text{CO}_2}$  is increased, one would expect, according to the carbamate hypothesis, that the greater the initial concentration of the available base in a given solution the greater should be the maximum carbamate in that solution. In Table II we show that to be the case, for here are given the maximum ratios of

TABLE I  
*Carbamate at or below Isoelectric pH of Hemoglobin*

| <i>t</i>            | Equilibrium   | (Hb)             | $P_{\text{CO}_2}$ at equilibrium | pH   | $\frac{(\text{HbAm}^-)}{(\text{Hb})}$ |
|---------------------|---------------|------------------|----------------------------------|------|---------------------------------------|
| $^{\circ}\text{C.}$ |               | <i>mm per l.</i> | <i>mm. Hg</i>                    |      |                                       |
| 0                   | Non-carbonate | 14 (Reduced)     | 92                               | 6.74 | 0.01                                  |
| 0                   | "             | 14 "             | 181                              | 6.38 | 0.25                                  |
| 0                   | "             | 14 "             | 276                              | 6.17 | 0.06                                  |
| 38                  | Total         | 9.1 "            | 475                              | 6.70 | 0.00                                  |
| 38                  | "             | 9.1 (Oxy-)       | 532                              | 6.62 | 0.00                                  |

TABLE II  
*Maximum  $(\text{HbAm}^-)/(\text{Hb})$  in Non-Carbonate Equilibrium of Hemoglobin As  $(B^+)/(\text{Hb})$  Increases*

$t = 0^{\circ}$ .

| (Hb)             | $\frac{(B^+)}{(\text{Hb})}$ | $\frac{(\text{HbAm}^-)}{(\text{Hb})}$ |
|------------------|-----------------------------|---------------------------------------|
| <i>mm per l.</i> |                             |                                       |
| 10 (Oxy-)        | 0.3                         | 0.3                                   |
| 10 (Reduced)     | 2.8                         | 1.2                                   |
| 18 "             | 3.7                         | 1.2                                   |
| 10 "             | 4.2                         | 2.0                                   |
| 15 "             | 4.9                         | 1.8                                   |
| 10 (Oxy-)        | 5.8                         | 3.4                                   |
| 10 (Reduced)     | 5.9                         | 3.9                                   |
| 10 (Oxy-)        | 5.9                         | 3.0                                   |

carbamate to hemoglobin in a number of hemoglobin solutions containing increasing quantities of available base; and it is seen that these ratios increase as the ratios of available base to hemoglobin of each solution increase.

4. In the case of simple amino acids, the rate of formation of carbamate is rapid. The same rapid formation is demonstrable

in the case of hemoglobin in the following way, as Meldrum and Roughton (1933) first found, and as we have confirmed. When carbonic anhydrase, the enzyme which catalyzes the hydration of  $\text{CO}_2$  to  $\text{H}_2\text{CO}_3$  in hemoglobin solutions, is inhibited by KCN, rapid formation of carbonates cannot occur; nevertheless a rapid uptake of  $\text{CO}_2$  from a gas phase is observed; and this is due solely to the formation of carbamate. Typical experiments are discussed in the section on the non-carbonate equilibrium.

In the case of serum proteins also, the same rapid formation of carbamate (see Fig. 7) was found by us. This is evidence that the carbamate hypothesis is applicable in this case likewise.

5. The heat of formation of carbamate in the case of simple amino acids is high and positive. That the same is true in the case of hemoglobin will be shown later.

6. Finally, equilibrium equations derived on the basis of the carbamate hypothesis are in excellent agreement with all the experimental facts in the literature and new experiments designed to test them.

*Two Types of Equilibria*—In Paper I it was shown that simple amino acids may give two kinds of equilibrium with  $\text{CO}_2$ . These two are also found in the case of hemoglobin, and are now discussed.

*Non-Carbonate Equilibrium*—The addition of 0.05 to 0.1 M cyanide to a hemoglobin solution inhibits completely the catalysis by carbonic anhydrase of the hydration of  $\text{CO}_2$  to  $\text{H}_2\text{CO}_3$ . Under these circumstances a hemoglobin solution equilibrated with gaseous  $\text{CO}_2$  takes up the  $\text{CO}_2$  in two phases: (1) an initial rapid phase, due mainly to the rapid formation of carbamate; (2) a subsequent slow phase, particularly slow at  $0^\circ$ , due entirely to the slow uncatalyzed hydration of  $\text{CO}_2$  to  $\text{H}_2\text{CO}_3$ . At the end of the first phase, carbamate formation is complete and carbonate is virtually absent. In effect, an equilibrium is established involving hemoglobin, carbamate, and  $\text{CO}_2$  only; this we call the "non-carbonate" equilibrium. The method of studying the non-carbonate equilibrium, together with the mode of calculation of the carbamate concentration and the partial pressure of  $\text{CO}_2$  at equilibrium, was outlined in Paper I.

The typical biphasic curves of  $\text{CO}_2$  uptake in the case of hemoglobin are in all respects analogous to that of glycine shown in Fig. 4 of Paper I.

*Total Equilibrium*—When cyanide-free solutions of hemoglobin are equilibrated with  $\text{CO}_2$  by the usual tonometric method (Austin *et al.*, 1922), no such division into a rapid and slow phase is possible because the  $\text{CO}_2$  is rapidly hydrated to  $\text{H}_2\text{CO}_3$ . Bicarbonate as well as carbamate is present; the equilibrium is the "total" equilibrium. (In this case, one can determine the carbamate by the Ferguson-Roughton (1934) method, the carbonate by difference from the total bound  $\text{CO}_2$ , the pH by the glass electrode, the total  $\text{CO}_2$  by the Van Slyke-Neill (1924) method, and the  $P_{\text{CO}_2}$  at equilibrium by calculation from the  $\text{CO}_2$  of the gas phase.)

The advantage of distinguishing the non-carbonate from the the total equilibrium lies in the fact that all changes in the former are due to carbamate formation only and that the equations which are to be applied in this case are simpler. Further, each equilibrium gives an independent method of testing the validity of the carbamate hypothesis. The non-carbonate equilibrium, however, has only physicochemical significance, whereas the total equilibrium has, in addition, physiological meaning.

*Derivation of Mass Action Equation for Carbamate Equilibrium*—The two necessary assumptions for the development of the equations in the case of hemoglobin are analogous to those made in the case of the simple amino acids: (1)  $\text{CO}_2$  combines directly with the amphanion ( $\text{Hb}^-$ ) only. This has been discussed above. (2) Exactly 1 equivalent of hydrogen ions is ionized for every mole of  $\text{CO}_2$  bound as carbamate. Accordingly, the equation of the reaction is



The combination is a hemoglobin carbamate. In customary parlance, it "binds" 1 equivalent of base per mole of  $\text{CO}_2$  combined as carbamate; therefore, it is properly designated by the symbol  $\text{HbAm}^-$  which, accordingly, denotes 1 mole of carbamate- $\text{CO}_2$ , capable of binding 1 equivalent of base. This second assumption, originally suggested by Henriques (1931), is of prime importance in the treatment of the carbamate equilibrium. It has never been experimentally proved. The proof is given in the next section, but anticipating this proof we may write the mass action equation for the carbamate equilibrium of hemoglobin and  $\text{CO}_2$  thus

$$\frac{(\text{H}^+)(\text{HbAm}^-)}{\alpha_{\text{CO}_2} P_{\text{CO}_2} (\text{Hb}^-)} = K_{\text{Am}} \quad (2)$$

*Distribution of Available Base in Carbamate Equilibrium*—Initially, in an alkaline hemoglobin solution, the equivalents of total available base are equal to the equivalents of hemoglobinate present; i.e.,  $(\text{B}^+) = (\text{Hb}^-)$ . In the non-carbonate equilibrium, there is 1 less mole of  $\text{Hb}^-$  for every mole of  $\text{CO}_2$  bound as carbamate; therefore, the base is distributed between the two forms according to the equation

$$(\text{B}^+) = (\text{Hb}^-) + (\text{HbAm}^-) \quad (3-a)$$

The concentration of hemoglobinate ( $\text{Hb}^-$ ) is given by the hemoglobin buffer Equation 1, whereas the concentration of the carbamate is given by the carbamate equilibrium Equation 2. In the total equilibrium bicarbonate must also be included, and the total available base is then given by the equation

$$(\text{B}^+) = (\text{Hb}^-) + (\text{HbAm}^-) + (\text{HCO}_3^-) \quad (3-b)^*$$

The concentration of  $(\text{HCO}_3^-)$  is given by the bicarbonate equilibrium equation

$$\frac{(\text{H}^+)(\text{HCO}_3^-)}{\alpha_{\text{CO}_2} P_{\text{CO}_2}} = K'_{\text{CO}_2} \quad (4)^*$$

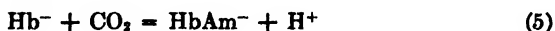
Equations 2 and 3-a (or Equation 3-b) are a restatement of the assumptions made; together with Equations 1 and 4, which are totally independent of any hypothesis of carbamate formation, they constitute a complete description of either the non-carbonate or the total equilibrium. The mass action constants of these

\* Most of our experimental work is at  $\text{pH} < 9$ , where  $\text{B}_2\text{CO}_3$  is negligible; we therefore leave it out of consideration.

\* Ordinarily this equation is written with the constant  $K'_1$ , loosely called the first dissociation constant of carbonic acid. It is now well established that the total free  $\text{CO}_2$  ( $= \alpha_{\text{CO}_2} P_{\text{CO}_2}$ ) at equilibrium is about  $1000 \times$  greater than that hydrated to  $\text{H}_2\text{CO}_3$ . In other words,  $K'_1$ , which is given by the equation  $((\text{H}^+)(\text{HCO}_3^-))/(\text{H}_2\text{CO}_3) = K'_1$  is  $1000 \times$  greater than  $K'_{\text{CO}_2}$ . The symbol  $K'_1$  must then be reserved to express the equilibrium of the first dissociation of  $\text{H}_2\text{CO}_3$ , and its use to express the equilibrium of Equation 4 should be abandoned.  $K'_{\text{CO}_2}$  could appropriately be called the bicarbonate equilibrium constant.

equations ( $K_{Am}$ ,  $K'_{CO_2}$ ,  $\beta_{Hb}$ ,  $\alpha_{CO_2}$ ,  $pI$ ) are functions of the temperature and the thermodynamic environment; ( $Hb$ ) and ( $B^+$ ) are constant in any given solution. A knowledge of these constants enables us to calculate the concentrations at equilibrium of total bound  $CO_2$ , ( $Hb^-$ ), ( $HbAm^-$ ), ( $HCO_3^-$ ), and ( $H^+$ ) as a function of  $P_{CO_2}$  over a wide range of conditions.

*Change of ( $H^+$ ) Resulting from Combination of  $CO_2$  and Hemoglobin As Carbamate*—According to the carbamate hypothesis, the reaction of  $CO_2$  with hemoglobin is



The nature of the change of ( $H^+$ ) in a hemoglobin solution when it combines with  $CO_2$  as carbamate is best studied in the non-carbonate equilibrium. Catalysis of hydration by carbonic anhydrase is inhibited by KCN; the amount of bicarbonate formed during the 1st half minute of the equilibration with  $CO_2$  is negligible; but carbamate formation is complete in this time; therefore, all pH changes are due to carbamate formation only.

Accordingly, the pH in the non-carbonate equilibrium was determined in the following way: A hemoglobin solution from isoelectric crystals containing 0.05 to 0.1 M cyanide (added as KCN with 1.00 equivalent of HCl) and known quantities of available base was equilibrated for 30 seconds with  $CO_2$  at known initial pressures. (The equilibrating apparatus was described in Paper I.) The solution was then rapidly removed from the apparatus and transferred to a glass electrode at the same temperature, and the pH was determined within 1 minute from the beginning of the equilibration; hence it is the pH of the non-carbonate equilibrium. The total  $CO_2$  was determined by analysis of the solution, the free  $CO_2$  was calculated from the  $P_{CO_2}$  at equilibrium, and the carbamate was calculated by difference.

Combining Equations 1 and 3-a, we get

$$\frac{(HbAm^-)}{(Hb)} = \frac{(B^+)}{(Hb)} - \beta_{Hb}(pH - pI) \quad (6)$$

Accordingly, one should find in the non-carbonate equilibrium (1) a linear relation between  $(HbAm^-)/(Hb)$  and pH; (2) a slope,  $\beta_{Hb}$ , equal to the buffer value ( $2.5 \pm 0.12$ ) of hemoglobin as determined by Van Slyke and coworkers (1922) by titration with HCl; (3) a

value of  $pI$  (that value of  $pH$  where  $(HbAm^-)/(Hb)$  equals  $(B^+)/(Hb)$ ) equal to the calculated value at  $0^\circ$  (approximately 7.5).<sup>4</sup> All these expectations were fulfilled, as shown by the data given in Fig. 1, in our experiments on beef and horse hemoglobin. Furthermore in Table III we give the values of  $\beta_{Hb}$  calculated from the data of experiments similar to the one outlined; these are in close agreement with the expected value. This evidence is proof that Equation 5 expresses correctly the course of the reaction, and that carbamate binds 1 equivalent of base per mole of  $CO_2$ , as given by Equations 3-a and 3-b.

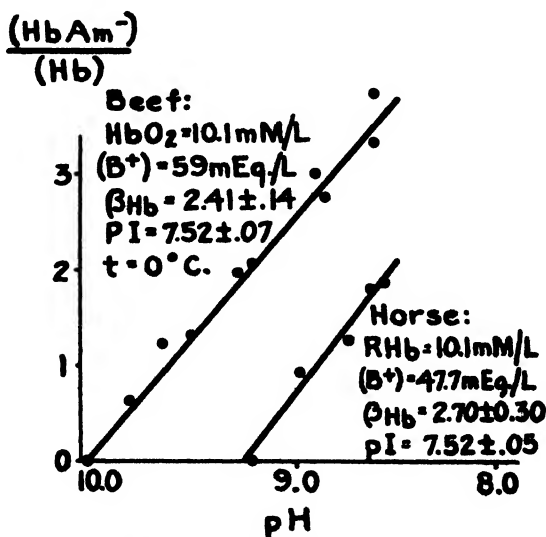


FIG. 1. Carbamate as a function of  $pH$  in the non-carbonate equilibrium of beef and horse hemoglobin at  $0^\circ$ .

*Distribution of Base in Total Equilibrium*—In the total equilibrium the concentration of the total bound  $CO_2$  (designated  $(\overline{CO_2})$ ) is equal to the sum of the concentrations of bicarbonate and carbamate. According to the carbamate hypothesis, the  $CO_2$  as carbamate binds an equivalent of base, as does also the bicarbonate; therefore the available base according to Equation 3-b is

$$(B^+) = (Hb^-) + (\overline{CO_2}) \quad (7)$$

<sup>4</sup> Calculated from the value of  $pI = 6.8$  at  $38^\circ$  by the van't Hoff isochore,  $dpI/dt = Q/RT^2$ .  $Q$ , the heat of ionization of protein as an acid is  $-10,000$  calories (Stadie and Martin, 1924).



TABLE III

Observed Values of  $\beta_{\text{Hb}}$  from Slope of  $(\text{HbAm}^-)$ -pH Line in Non-Carbonate Equilibrium of Hemoglobin

| $t$       | (Hb)           | $\beta_{\text{Hb}}$ |
|-----------|----------------|---------------------|
| *C.       | mm per l.      |                     |
| 0         | 10.9 (Oxy-)    | 1.90 $\pm$ 0.20*    |
| 0         | 9.9 (Reduced)  | 3.64 $\pm$ 0.16     |
| 0         | 10.1 (Oxy-)    | 2.24 $\pm$ 0.25     |
| 0         | 10.1 (Reduced) | 2.70 $\pm$ 0.30     |
| 0         | 10.1 (Oxy-)    | 1.72 $\pm$ 0.22     |
| 0         | 10.4 "         | 2.79 $\pm$ 0.36     |
| 0         | 10.2 "         | 2.41 $\pm$ 0.14     |
| 25        | 10.4 "         | 2.79 $\pm$ 0.36     |
| 25        | 10.1 (Reduced) | 3.89 $\pm$ 0.23     |
| Mean..... |                | 2.67 $\pm$ 0.22     |

\* All deviation measures in this paper are standard errors rather than probable errors. P.E. = 0.6745 S.E. The methods of statistical analysis used are those outlined by Dunn (1929).

TABLE IV

Comparison of HCl and CO<sub>2</sub> Titration Curves of Beef Oxyhemoglobin

$t = 38^\circ$ ; (Hb), 18.1 mm per kilo; available base, 45.5 milli-equivalents per kilo.

| HCl  |                    | CO <sub>2</sub> |                              |
|------|--------------------|-----------------|------------------------------|
| pH   | (Hb <sup>-</sup> ) | pH              | ( $\overline{\text{CO}_2}$ ) |
|      | m.-eq. per kg.     |                 | mm per kg.                   |
| 7.65 | 45.5               | 7.52            | 5.7                          |
| 7.58 | 41.4               | 7.44            | 7.9                          |
| 7.24 | 32.4               | 7.40            | 9.5                          |
| 7.14 | 25.4               | 7.36            | 10.7                         |
| 6.93 | 16.4               | 7.32            | 12.1                         |
| 6.71 | 4.4                | 7.22            | 16.1                         |
| 6.67 | 3.2                | 7.11            | 22.6                         |
|      |                    | 7.04            | 25.3                         |

Statistical Analysis— $(\text{Hb}^-) = 2.37 (\text{Hb})[\text{pH} - 6.58] \pm 0.10$ .  $(\text{CO}_2)_{\text{obs.}} = (\text{B}^+) - 2.29 (\text{Hb})[\text{pH} - 6.54] \pm 0.04$ .  $(\text{CO}_2)_{\text{obs.}} - (\text{CO}_2)_{\text{calc.}} = -0.5 \pm 0.11$ .

By this equation one can calculate  $(\overline{\text{CO}_2})$  at any pH, since  $(\text{B}^+)$  is known and  $(\text{Hb}^-)$  can be calculated by Equation 1. (The

constants, *i.e.*  $\beta_{\text{Hb}}$  and  $pI$ , of Equation 1 can be determined by titration of the sodium hemoglobinate solution with HCl.) On the same solution one can measure, after equilibration with  $\text{CO}_2$ , ( $\overline{\text{CO}_2}$ ), and pH, and one can compare the observed value of ( $\overline{\text{CO}_2}$ ) with that calculated. If these values agree, there is a 1:1 equivalence of carbamate and base bound by it; if the observed ( $\overline{\text{CO}_2}$ ) exceeds that calculated, part or all of the carbamate does not bind base.

This experiment has been reported before, but never with independent pH determinations on both HCl and  $\text{CO}_2$  titration curves of the hemoglobin solutions (Hastings, Sendroy, Murray, and Heidelberger, 1924) nor with sufficient precision to escape criticism (Stadie and O'Brien, 1931). New data are given in Table IV. From the statistical analysis of these data, it is found throughout the experimental range of pH that  $(\overline{\text{CO}_2})_{\text{obs.}} - (\overline{\text{CO}_2})_{\text{calc.}} = -0.5 \pm 0.1$  mM per kilo. The observed ( $\overline{\text{CO}_2}$ ) was slightly less than that calculated; since it was not more, we believe there was no significant difference between them. We conclude, therefore, as we did from our experiments on the non-carbonate equilibrium, that the carbamate present, which can be calculated to have an average concentration of 1.5 mM per kilo, binds exactly 1 equivalent of base.

The identity of the  $\text{CO}_2$  titration curve with that of HCl was formerly considered as proving that all the bound  $\text{CO}_2$  was bicarbonate, and none was carbamate. Henriques (1931), however, pointed out the possibility that carbamate, just like bicarbonate, might bind base equivalent to its  $\text{CO}_2$ ; in that case the base other than hemoglobinate would be precisely equal to the total bound  $\text{CO}_2$ , and the observed and calculated  $\text{CO}_2$  should be equal. In other words, this experiment by itself could not show whether or not carbamate existed; but the proof, by independent direct analysis, that it does exist in hemoglobin solutions and the proof, given above, that the  $\text{CO}_2$  and HCl titration curves are identical show that Henriques was correct in his surmise.

*Mass Action Constant in Non-Carbonate Equilibrium*—We determined ( $\text{HbAm}^-$ ) and  $P_{\text{CO}_2}$  in the non-carbonate equilibrium of a series of solutions of hemoglobin, either in the oxy- or reduced form, containing known quantities of available base. We calculated ( $\text{Hb}^-$ ) by Equation 3-a and pH by Equation 1. The variables in Equation 2 slightly transformed, *viz.*

$$\frac{(\text{HbAm}^-)}{(\text{Hb}^-)} = \frac{\alpha_{\text{CO}_2} P_{\text{CO}_2} K_{\text{Am}}}{(\text{H}^+)} \quad (8)$$

were then known, and  $K_{\text{Am}}$  was calculated in the following way:  $(\text{HbAm}^-)/(\text{Hb}^-)$  was plotted against  $\alpha_{\text{CO}_2} P_{\text{CO}_2}/(\text{H}^+)$ . These quantities, according to the equation, should be in linear relationship to each other; this was found to be the case, as shown by Fig. 2. The slope of the line, which is  $10^6 K_{\text{Am}}$ , and its standard error were statistically calculated and are included in the data.

In Fig. 3 are given the plots of  $(\text{HbAm}^-)$  against  $P_{\text{CO}_2}$  in four

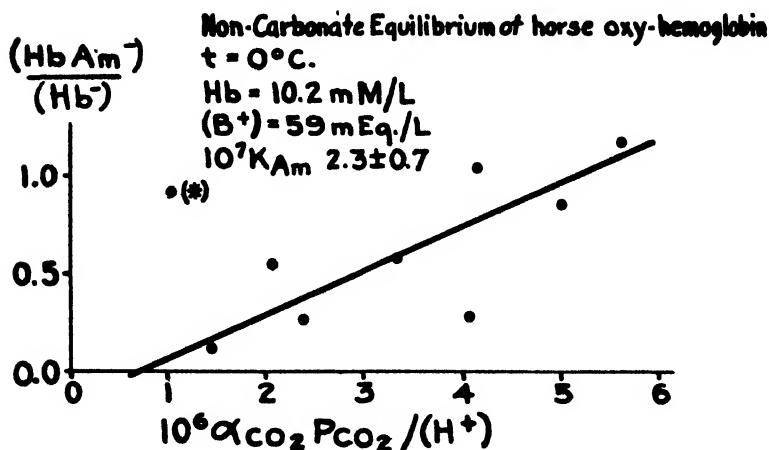


Fig. 2. Plot of the mass action equation  $(\text{HbAm}^-)/(\text{Hb}^-) = K_{\text{Am}} \alpha_{\text{CO}_2} P_{\text{CO}_2}/(\text{H}^+)$ . The slope of the line is  $K_{\text{Am}} \times 10^6$ .

solutions which varied in their hemoglobin concentrations and in their ratios of available base to hemoglobin. In each case the full line was calculated with the value of  $K_{\text{Am}}$  calculated as above outlined. In all cases the fit of the experimental points to the calculated line is good, and the form of the curve is the same as that first described by Meldrum and Roughton (1933) for the same type of equilibrium. Although the concentration of hemoglobin and base varied considerably, the values of the mass action constants are approximately the same. Fig. 3 also brings out the fact, already referred to, that as the ratio of  $(\text{B}^+)/(\text{Hb})$  increases,

the curves become steeper, and the maximum carbamate is greater. In Table V we give the values of  $K_{Am}$  in various hemoglobin solutions determined by the same method. The smallness of the standard error of  $K_{Am}$  in any one solution shows that the relative accu-

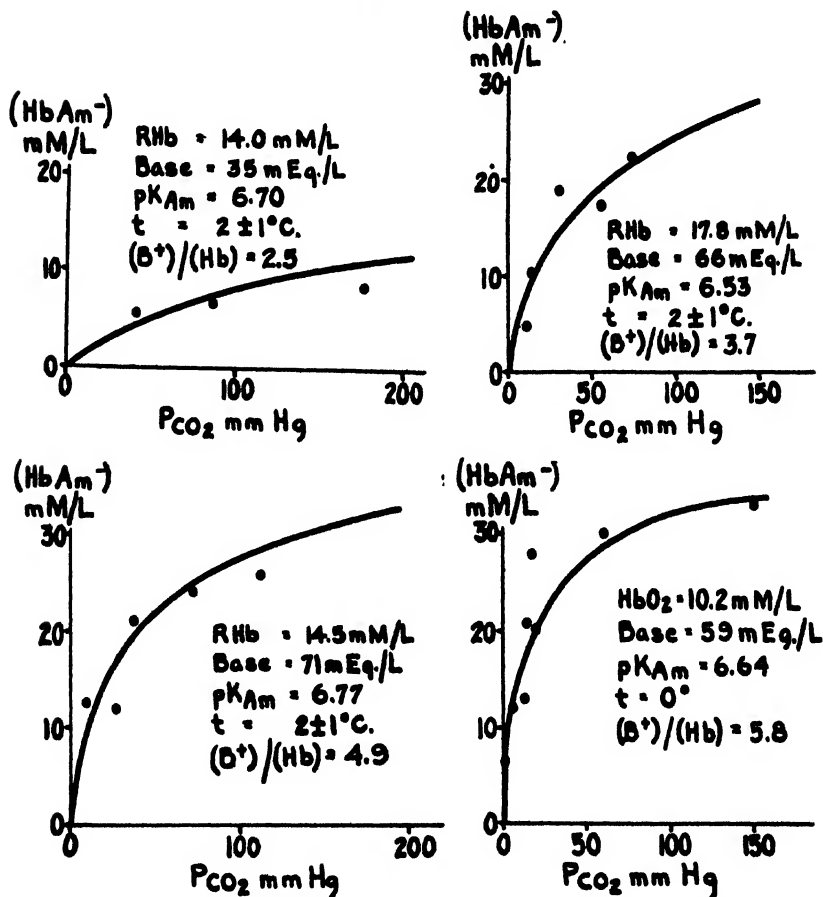


FIG. 3. The non-carbonate equilibrium of hemoglobin and CO<sub>2</sub> at 0°C. The full lines are calculated from the equilibrium constants.

racy of the method is good, but the variation of  $K_{Am}$  in the different solutions shows that the reproducibility of the method from solution to solution is not so good. In all cases the variations are within the limits of the experimental method, and we believe these

experiments show that Equation 2 correctly expresses the non-carbonate equilibrium of hemoglobin and  $\text{CO}_2$ .

*Mass Action Constant in Total Equilibrium*—By Equation 4,  $\alpha_{\text{CO}_2} P_{\text{CO}_2} = (\text{H}^+) (\text{HCO}_3^-) / K'_{\text{CO}_2}$ ; therefore by substitution Equation 2, which describes the total equilibrium also, becomes

$$\frac{(\text{HbAm}^-)}{(\text{HCO}_3^-)} = \frac{K_{\text{Am}}}{K'_{\text{CO}_2}} (\text{Hb}^-) \quad (9)$$

$(\text{HbAm}^-)$ ,  $(\text{HCO}_3^-)$ , and  $(\text{H}^+)$  may be determined by analysis;  $(\text{Hb}^-)$  may be calculated by Equation 7 from the known  $(\text{B}^+)$  and the total bound  $\text{CO}_2$ ;  $K'_{\text{CO}_2}$  may be calculated from the ionic strength of the solution by the equation  $\text{p}K'_{\text{CO}_2} = 6.33 - 0.5 \sqrt{\mu}$

TABLE V

*Carbamate Equilibrium Constant,  $K_{\text{Am}}$ , Calculated from Non-Carbonate Equilibrium of Hemoglobin Solutions*

| <i>t</i> | (Hb)                | $K_{\text{Am}} \times 10^7$ |
|----------|---------------------|-----------------------------|
| °C.      | <i>mm per l.</i>    |                             |
| 0        | 10 (Human, reduced) | $1.5 \pm 0.3$               |
| 0        | 10 (Beef, oxy-)     | $2.3 \pm 0.7$               |
| 0        | 11 (Horse, reduced) | $7.4 \pm 0.2$               |
| 0        | 11 ( " oxy-)        | $4.1 \pm 1.5$               |
| 0        | 10 ( " reduced)     | $2.2 \pm 0.3$               |
| 0        | 10 " "              | $4.1 \pm 1.0$               |
| 0        | 10 ( " oxy-)        | $3.3 \pm 0.5$               |
| 25       | 14 ( " reduced)     | $43 \pm 2$                  |
| 25       | 14 ( " oxy-)        | $30 \pm 4$                  |

at  $38^\circ$ ; therefore  $K_{\text{Am}}$  may be calculated. In Table VI we give the experimental data on a hemoglobin solution in the reduced and oxygenated form. From Equation 9 we should expect  $(\text{HbAm}^-)/(\text{HCO}_3^-)$  to be in linear relation with  $(\text{Hb}^-)$ ; this is found to be the case, as shown by the plot of the data in Fig. 4. The slope of the line is  $K_{\text{Am}}/K'_{\text{CO}_2}$ , which, together with its standard error, we have calculated statistically. In Figs. 5, *a* and 5, *b*, from the data of Table VI, are given plots of  $(\text{HbAm}^-)$  against pH and also against  $P_{\text{CO}_2}$ . The curves were calculated from the values of  $K_{\text{Am}}/K'_{\text{CO}_2}$  given in Fig. 4 and the constants  $(\text{B}^+)$ ,  $(\text{Hb})$ ,  $\alpha_{\text{CO}_2}$ ,

TABLE VI

*Total Equilibrium of Reduced and Oxyhemoglobin and Carbon Dioxide*  
 $t = 38^\circ$ ; (Hb) = 9.1 mm per liter; available base = 45.3 milli-equivalents per liter; NaCl = 125.0 mm per liter;  $\alpha_{\text{CO}_2} = 2.9 \times 10^{-3}$  M per. liter per mm. of Hg;  $\text{pK}'_{\text{CO}_2}$  (calculated) = 6.13.

| $P_{\text{CO}_2}$ | pH   | Total $\text{CO}_2$ | $(\text{HCO}_3^-)$ | $(\text{HbAm}^-)$ |
|-------------------|------|---------------------|--------------------|-------------------|
| Reduced Hb        |      |                     |                    |                   |
| mm. Hg            |      | mm per l.           | mm per l.          | mm per l.         |
| $\pm 0^*$         | 8.81 | 0.7                 | 0.0                | 0.65              |
| $\pm 0^*$         | 8.56 | 3.3                 | 1.8                | 1.48              |
| $\pm 0^*$         | 8.54 | 3.6                 | 2.2                | 1.43              |
| 0.7               | 8.52 | 6.7                 | 4.9                | 1.77              |
| 2.3               | 8.15 | 10.1                | 8.0                | 2.05              |
| 10.2              | 7.82 | 16.6                | 13.6               | 2.71              |
| 47.4              | 7.39 | 27.7                | 23.3               | 3.43              |
| 139               | 7.11 | 39.5                | 33.4               | 2.11              |
| 271               | 6.89 | 50.3                | 40.9               | 1.37              |
| 475               | 6.70 | 60.0                | 46.3               | $\pm 0.0$         |
| Oxyhemoglobin     |      |                     |                    |                   |
| 3.6*              | 7.91 | 8.3                 | 6.8                | 1.42              |
| 10.6              | 7.70 | 12.2                | 10.5               | 1.36              |
| 25.0              | 7.51 | 17.3                | 15.2               | 1.41              |
| 53.0              | 7.30 | 23.0                | 20.0               | 1.48              |
| 96.0              | 7.11 | 30.4                | 26.2               | 1.40              |
| 177               | 6.99 | 39.2                | 33.1               | 0.98              |
| 312               | 6.78 | 47.6                | 38.1               | 0.51              |
| 532               | 6.62 | 59.7                | 44.3               | 0.00              |

## Statistical Analysis

|                         | $K_{\text{Am}}/K'_{\text{CO}_2}$ | $K_{\text{Am}} \times 10^6$ |
|-------------------------|----------------------------------|-----------------------------|
| Reduced hemoglobin..... | $8.6 \pm 1.1$                    | $6.4 \pm 0.80$              |
| Oxyhemoglobin.....      | $3.4 \pm 0.47$                   | $2.5 \pm 0.31$              |

\* Excluded from calculation of  $K_{\text{Am}}$ .

$\beta_{\text{CO}_2}$ , and  $\text{pI}^5$  The curves are of the same general type as those found in the case of the total equilibrium of the simple amino acids.

<sup>5</sup> The calculation of the full curves was made as follows: Assume a pH; then (Hb<sup>-</sup>) is given by Equation 1, ( $\text{CO}_2$ ) by Equation 7, (HbAm<sup>-</sup>) by Equation 9 in the form

$$\frac{(\text{HbAm}^-)}{(\text{CO}_2) - (\text{HbAm}^-)} = \frac{K_{\text{Am}}}{K_{\text{CO}_2}} (\text{Hb}^-)$$

( $\text{HCO}_3^-$ ) by difference of ( $\text{CO}_2$ ) and (HbAm<sup>-</sup>), and  $P_{\text{CO}_2}$  by Equation 4. The constants used are (Hb) = 9.1 mm per liter,  $\alpha_{\text{CO}_2} = 2.9 \times 10^{-3}$  mm per

They show no carbamate at high pH (since  $P_{\text{CO}_2}$  is 0), a maximum carbamate at intermediate pH (at very low  $P_{\text{CO}_2}$ ), and again no carbamate at isoelectric pH. In the physiological range, the curves are practically flat, but the reduced curve is always higher than the oxygenated one, points which will be commented on later.

The agreement of the experimental points with the calculated curve leads us to conclude that the theory outlined expresses correctly the total carbamate equilibrium.

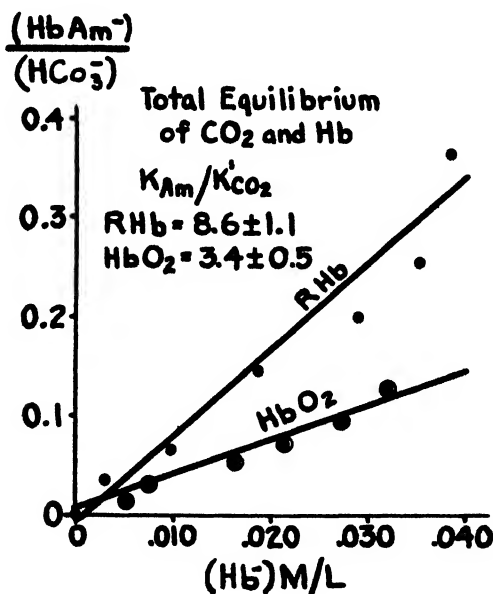


FIG. 4. Plot of the mass action equation  $(\text{HbAm}^-)/(\text{HCO}_3^-) = (\text{Hb}^-)K_{\text{Am}}/K'_{\text{CO}_2}$  in the total equilibrium. The slope of the line is  $K_{\text{Am}}/K'_{\text{CO}_2}$ .

*Ratio of  $K_{\text{Am}}$  in Reduced and Oxyhemoglobin*—The carbamate of reduced hemoglobin, both in the non-carbonate and the total equilibrium, is always higher than that of oxyhemoglobin. There-

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liter per mm. of Hg of  $P_{\text{CO}_2}$ . For reduced hemoglobin  $\text{pI} = 6.7$ ,  $\beta_{\text{Hb}} = 2.44$ ,  $\text{pK}'_{\text{CO}_2} = 6.14$ ,  $K_{\text{Am}}/K'_{\text{CO}_2} = 8.6$ ,  $(\text{B}^+) = 45.3$  milli-equivalents per liter. For oxyhemoglobin  $\text{pI} = 6.6$ ,  $\beta_{\text{Hb}} = 3.1$ ,  $\text{pK}'_{\text{CO}_2} = 6.14$ ,  $K_{\text{Am}}/K'_{\text{CO}_2} = 3.4$ ,  $(\text{B}^+) = 43.9$  milli-equivalents per liter.

fore, one should expect the value of the carbamate constant in reduced hemoglobin to be greater than in oxyhemoglobin; *i.e.*, the ratio  $K_{Am(r)}/K_{Am(o)} > 1$ . This was found to be the case in experiments which were done especially to measure this ratio,

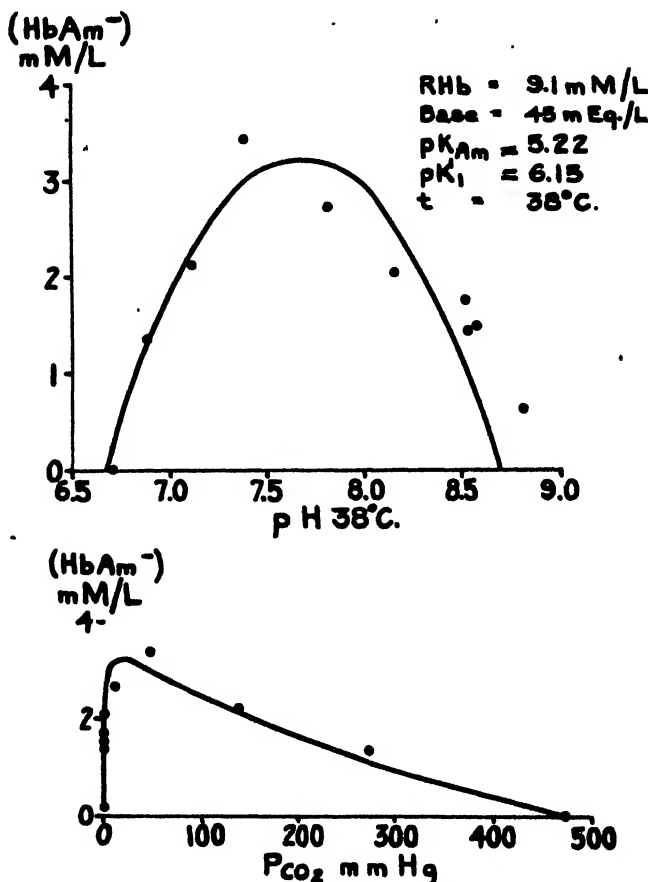


FIG. 5, a. The total carbamate equilibrium of reduced hemoglobin at  $38^\circ$ . The full lines are calculated from the equilibrium constants.

and which are shown in Table VII. There is also included a value of this ratio calculated from the data of Meldrum and Roughton.

*Calculation of Difference of Hemoglobin Carbamate of Reduced and Oxyhemoglobin at Constant pH from  $K_o$  and  $K_r$ , Acid Dissocia-*



*tion Constants of Oxylabile Group of Hemoglobin*—The work of Van Slyke and coworkers (1922) has demonstrated that the combination of oxygen with reduced hemoglobin results in an ionization of  $H^+$ ; but the combination as carbamate of  $CO_2$  with hemoglobin also ionizes  $H^+$ ; therefore the two reactions, *viz.* (1)  $Hb + O_2 = HbO_2 + H^+$  and (2)  $Hb + CO_2 = HbCO_2 + H^+$ ,

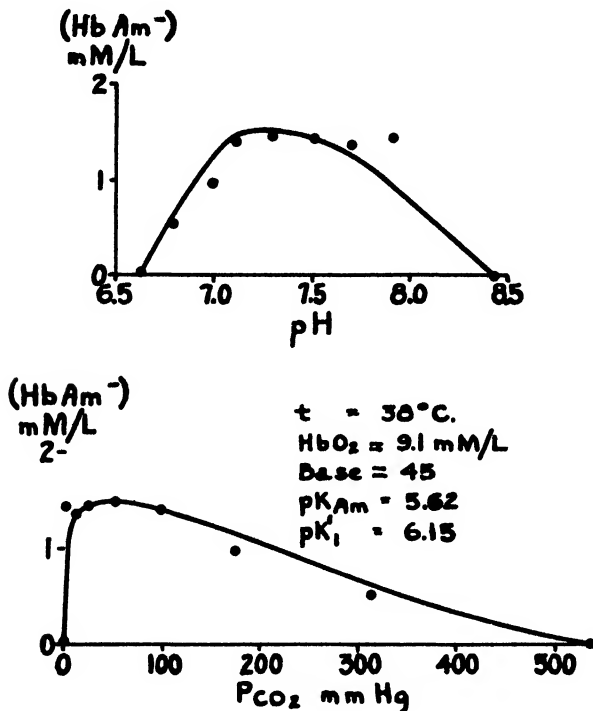


FIG. 5, b. The total carbamate equilibrium of oxyhemoglobin at  $38^\circ$ . The full lines are calculated from the equilibrium constants.

should oppose each other. Accordingly, one should find (1) that hemoglobin containing carbamate binds oxygen less readily than does hemoglobin containing none, and (2) that oxyhemoglobin binds  $CO_2$  as carbamate less readily than does reduced hemoglobin. Both of these expectations have been experimentally verified: Margaria and Green (1933) found a lowered oxygen saturation when a solution of hemoglobin contained appreciable carbamate.

and Meldrum and Roughton (1933) first showed that oxyhemoglobin binds less carbamate than reduced hemoglobin.

We have amply confirmed this latter fact, as the data of this paper show; but in addition we shall show that the difference of carbamate in reduced and oxyhemoglobin is calculable on the basis of our second assumption alone and the currently accepted theory of the base-binding properties of oxyhemoglobin and reduced hemoglobin. According to this theory, as developed by Van Slyke and coworkers (1922), the acid groups of hemoglobin which bind

TABLE VII  
Ratio of  $K_{Am}$  (Reduced Hb) to  $K_{Am}$  (Oxy-Hb)

| <i>t</i>  | Equilibrium   | $\frac{K_{Am(r)}}{K_{Am(o)}}$ |
|-----------|---------------|-------------------------------|
| °C.       |               |                               |
| 0         | Non-carbonate | $1.3 \pm 0.4$                 |
| 25        | "             | $1.4 \pm 0.2$                 |
| 0, 15*    | "             | $1.7 \pm 0.3$                 |
| 38        | Total         | $2.5 \pm 0.5$                 |
| Mean..... |               | $1.7 \pm 0.3$                 |

\* Calculated from Meldrum and Roughton ((1933) Fig. 6) as follows: From Equation 2 at constant  $(HbAm^-)$ , therefore constant  $(Hb^-)$ ,

$$\frac{K_{Am(r)}}{K_{Am(o)}} = \frac{(H^+)_r}{(H^+)_o} \cdot \frac{PCO_2(o)}{PCO_2(r)}$$

But at constant  $(Hb^-)$  we know that  $(H^+)_r/(H^+)_o \simeq 10^{-0.3} \simeq 0.63$ . From Fig. 6 of Meldrum and Roughton we estimate from the curves at the two temperatures that  $PCO_2(o)/PCO_2(r) = 2.7 \pm 0.6$ . Then  $K_{Am(r)}/K_{Am(o)} = 1.7 \pm 0.3$ .

base are of two types. (1) There is a single acid group which is affected by oxygenation; in oxyhemoglobin its acid dissociation constant ( $K_o$ ) is greater than the corresponding acid dissociation constant ( $K_r$ ) of reduced hemoglobin. This characteristic of the "oxylabile" acid group, as it has been called, makes oxyhemoglobin a stronger acid than reduced hemoglobin. The base bound by this group is equal to  $\frac{K_o}{K_o + (H^+)} (Hb)$  in the case of oxyhemoglobin, and  $\frac{K_r}{K_r + (H^+)} (Hb)$  in the case of reduced hemoglobin.

(2) There are many oxystable groups unaffected by oxygenation. The base bound by them is here designated  $(\text{Hb}^-)$ .

Accordingly, in the non-carbonate equilibrium of a hemoglobin solution with  $\text{CO}_2$ , the total available base may be written

$$(\text{B}^+) = \frac{K_o}{K_o + (\text{H}^+)} (\text{Hb}) + (\text{Hb}^-) + (\text{HbAm}^-).$$

or 
$$(\text{B}^+) = \frac{K_r}{K_r + (\text{H}^+)} (\text{Hb}) + (\text{Hb}^-) + (\text{HbAm}^-),$$

TABLE VIII

*Comparison in Non-Carbonate Equilibrium of Observed Difference of Carbamate at Constant pH between Reduced and Oxyhemoglobin Solutions with That Calculated by Equation 10*

| (Hb)   | Available base       | pH   | $\frac{(\text{HbAm}^-)_r - (\text{HbAm}^-)_o}{(\text{Hb})}$ |            |
|--|----------------------|------|---|------------|
|  |                      |      | Observed  | Calculated |
| $t = 0^\circ; \text{pK}_o = 7.39; \text{pK}_r = 8.89$  |                      |      |   |            |
| <i>mM per l.</i>                                       | <i>m.-eq. per l.</i> |      |   |            |
| 12 1   | 36                   | 8 31 | 0 29  | 0 68       |
|  |                      | 8 17 | 0 34  | 0 68       |
|  |                      | 8 09 | 0 40  | 0 69       |
|  |                      | 8 04 | 0 55  | 0 68       |
|  |                      | 7 90 | 0 44  | 0 67       |
|  |                      | 7 86 | 0 66  | 0 66       |
| 10 1   | 52                   | 8 98 | 0 50  | 0 42       |
|  |                      | 8 94 | 0 23  | 0 54       |
|  |                      | 8 02 | 0 58  | 0 60       |
|  |                      | 7 56 | 0 53  | 0 62       |
| $t = 25^\circ; \text{pK}_o = 6.85; \text{pK}_r = 8.35$ |                      |      |   |            |
| 10 0   | 40                   | 7 87 | 0 51  | 0 68       |
|  |                      | 7 70 | 0 61  | 0 68       |

in the respective cases of oxyhemoglobin and reduced hemoglobin. For the same solution of hemoglobin, since  $(\text{B}^+)$  is constant, we have at constant pH (for then  $(\text{Hb}^-)$ , is unchanged)

$$\frac{(\text{HbAm}^-)_r - (\text{HbAm}^-)_o}{(\text{Hb})} = \frac{K_o}{K_o + (\text{H}^+)} - \frac{K_r}{K_r + (\text{H}^+)} \quad (10)$$

We should be able to calculate, using the values of  $K_o$  and  $K_r$  of horse hemoglobin determined by independent methods,<sup>6</sup> the value in the non-carbonate equilibrium of the ratio of the left of Equation 10. Such calculated values are compared with those observed in Table VIII. At higher pH values the agreement is not good. But this, in all probability, is attributable to low concentration of carbamate in both oxy- and reduced hemoglobin, a fact which magnifies greatly the errors of small differences. Much more weight must be given to the good agreement at low pH where carbamate concentration is greater and errors of differences are smaller. The experiment appears to show, therefore, that the difference of carbamate in reduced and oxyhemoglobin is a function of the ionization of the oxylabile group as expressed by Equation 10.

*Heat of Carbamate Reaction*—Meldrum and Roughton (1933) found, in experiments of a preliminary nature, that the directly measured heat of carbamate formation in the case of ammonia and glycine was positive and high; therefore, we should expect a similar value to be found in the case of hemoglobin. No direct calorimetric measurements are available, but from a comparison of the carbon dioxide tensions of two non-carbonate equilibrium curves at 0° and 15° Meldrum and Roughton estimated that the heat of the reaction is about 20,000 calories—a value of the order expected. More correctly than from the value of  $P_{CO_2}$  at equilibrium, we should calculate the heat of the reaction  $Hb^- + CO_2$  (gas) =  $HbAm^- + H^+$  from the equilibrium constants at two temperatures. We have made this calculation, using the van't Hoff equation

$$2.3 \log \frac{K_{Am}}{K'_{Am}} = \frac{Q}{R} \left( \frac{1}{T} - \frac{1}{T'} \right)$$

( $K_{Am}$  and  $K'_{Am}$  are the equilibrium constants at the respective temperatures  $T$  and  $T'$ .) In Table IX are shown the heats of the

<sup>6</sup> We used the values, determined by HCl titration of reduced and oxy-hemoglobin, given by Hastings, Van Slyke, Neill, Heidelberger, and Harrington (1924). At 38°  $pK_o = 6.70$  and  $pK_r = 8.13$ . These are corrected to the temperature of the experiment by the equation  $\frac{d \ln k}{dt} = -Q/RT^2$ , where  $-Q = 10,000$  calories, the heat of ionization of the protein acid as estimated by Stadie and Martin (1924).

reaction calculated from our experiments on glycine and hemoglobin. In two such dissimilar amino acids as these, the equilibrium constants are of the same order of magnitude, and the heats of the reaction are not significantly different—facts in agreement with the carbamate hypothesis.  $Q$ , as we have calculated it here, includes the heat of ionization of hemoglobin as an acid ( $-10,000$  calories) and the heat of solution of  $\text{CO}_2$  (4900 calories). Subtracting these heats algebraically, we get 17,000 calories for the heat of combination of  $\text{CO}_2$  with hemoglobin; *i.e.*,  $\text{Hb} + \text{CO}_2$  (aqueous) =  $\text{HbCO}_2 + 17,000$  calories.

$pK_{\text{CO}_2}^*$  in Hemoglobin Solutions—In simple aqueous solution

TABLE IX

*Heat Evolved in Carbamate Reaction Calculated from Equilibrium Constant at Two Temperatures*

| Substance                             | $t$ | $K_{\text{Am}} \times 10^6$ | $Q$ , calculated  |
|---------------------------------------|-----|-----------------------------|-------------------|
|                                       | °C. |                             |                   |
| Glycine (Stadie and O'Brien, 1935-36) | 0   | 1.6                         | 8,900 $\pm$ 3300  |
|                                       | 23  | 3.4                         |                   |
|                                       | 38  | 12.6                        |                   |
| Reduced Hb (data of this paper)       | 0   | 0.41                        | 12,000 $\pm$ 1000 |
|                                       | 38  | 6.1                         |                   |

the mass action equation for the equilibrium of  $\text{CO}_2$  and bicarbonate is

$$\frac{(\text{H}^+)(\text{HCO}_3^-)}{\alpha_{\text{CO}_2} P_{\text{CO}_2}} = K'_{\text{CO}_2} \quad (4)$$

$K'_{\text{CO}_2}$  is known to vary with the ionic strength ( $\mu$ ) of the solution, and Hastings and Sendroy (1925) have shown that at 38° the relation  $pK'_{\text{CO}_2} = 6.33 - 0.5\sqrt{\mu}$  expresses this variation with considerable precision. In a hemoglobin solution, however, an analogous equilibrium equation expressing the relations of the total bound  $\text{CO}_2$  (designated  $(\overline{\text{CO}_2})$ ),  $\text{H}^+$ , and  $P_{\text{CO}_2}$ , *viz.*,

$$\frac{(\text{H}^+)(\overline{\text{CO}_2})}{\alpha_{\text{CO}_2} P_{\text{CO}_2}} = K^*_{\text{CO}_2} \quad (11)$$

does not give a value of  $K_{\text{CO}_2}^*$ , corresponding to that calculated from the ionic strength equation. On the contrary, Van Slyke, Hasting, Murray, and Sendroy (1925) found that  $K_{\text{CO}_2}^*$  is much higher. Later, this alteration of  $K_{\text{CO}_2}^*$  from the expected value was studied in detail by Stadie and Hawes (1928) and Margaria and Green (1933); they found also that  $K_{\text{CO}_2}^*$  is greater in reduced hemoglobin than in oxyhemoglobin solutions of identical ionic strength. Henriques (1928) and later Margaria and Green (1933)

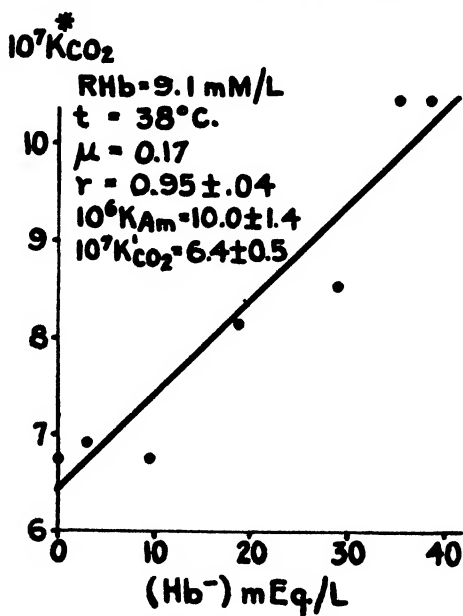


FIG. 6. The relation of  $K'_{\text{CO}_2}$  and (Hb<sup>-</sup>). The slope of the line is  $K_{\text{Am}}$ .  $r$  = correlation coefficient.

suggested that both the higher value of  $K_{\text{CO}_2}^*$  and its dependence on the state of the hemoglobin could be most simply explained by the presence of part of the total bound  $\text{CO}_2$  as carbamate. The direct determination of carbamate in hemoglobin solutions leaves little doubt that this explanation is correct.

The relation between  $K'_{\text{CO}_2}$  and  $K_{\text{CO}_2}^*$  is derived as follows: The total bound  $\text{CO}_2$  is expressed by the equation

$$(\overline{\text{CO}_2}) = (\text{HCO}_3^-) + (\text{HbAm}^-) \quad (12)$$

The bicarbonate is given by Equation 4; the carbamate is given by Equation 2; therefore, the combination of Equations 2, 4, 11, and 12 gives

$$K^*_{CO_2} = K'_{CO_2} + K_{Am}(Hb^-) \quad (13)$$

as the desired relationship.  $K^*_{CO_2}$  is thus seen to be a pseudo-constant dependent on two mass action constants,  $K'_{CO_2}$  and  $K_{Am}$ , and a variable,  $(Hb^-)$ , which itself is a function of hemoglobin, the available base, and the pressure of  $CO_2$  and oxygen. It will be shown that Equation 13 conforms with the experimental facts by

TABLE X

*Calculation of  $K_{Am}$  and  $K'_{CO_2}$  of Reduced Horse Hemoglobin at 38° from Data of Margaria and Green ((1933) Tables II and III)*

| Experiment No. | (Hb)             | $r^*$           | $K_{Am} \times 10^6$ | $\frac{K_{Am}}{K'_{CO_2}}$ | Ratio<br>$\frac{\text{Observed } K'_{CO_2}}{\text{Calculated } K'_{CO_2}}$ |
|----------------|------------------|-----------------|----------------------|----------------------------|--|
|                | <i>mm per l.</i> |                 |                      |                            |  |
| 2              | 3.2              | $0.94 \pm 0.07$ | $5.5 \pm 1.1$        | $9.3 \pm 1.9$              | 1.02   |
| 3              | 2.9              | $0.74 \pm 0.26$ | $3.9 \pm 2.0$        | $5.4 \pm 3.3$              | 0.91   |
| 5              | 8.8              | $0.92 \pm 0.11$ | $3.5 \pm 1.1$        | $5.1 \pm 1.9$              | 0.81   |
| 6              | 8.0              | $0.77 \pm 0.29$ | $2.5 \pm 1.5$        | $3.6 \pm 2.1$              | 1.01   |
| 7              | 16.0             | $0.97 \pm 0.03$ | $4.0 \pm 0.6$        | $5.0 \pm 0.7$              | 1.37   |
| 8              | 15.3             | $0.79 \pm 0.22$ | $1.4 \pm 0.6$        | $2.0 \pm 0.9$              | 1.01   |
| 9              | 12.6             | $0.99 \pm 0.01$ | $6.1 \pm 0.5$        | $8.3 \pm 0.7$              | 1.09   |
| Mean.....      |                  |                 | $3.8 \pm 0.5$        | $5.5 \pm 0.9$              | $1.03 \pm 0.04$  |

\* Correlation coefficient of  $K^*_{CO_2}$  and  $(Hb^-)$ .

using it to calculate  $K_{Am}$  and  $K'_{CO_2}$ . Fig. 6 is from our own data on the total equilibrium of beef hemoglobin at 38°. One sees, as is expected from Equation 13, that there is a linear relation between  $K^*_{CO_2}$  and  $(Hb^-)$ . Further, the slope of the line, *i.e.*  $K_{Am}$ , is  $(10 \pm 1.4) \times 10^{-6}$ —a value in substantial agreement with that in Table VI calculated from the directly determined carbamate. When  $(Hb^-) = 0$ , that is when no carbamate is present,  $K^*_{CO_2} = K'_{CO_2}$ . Accordingly, by extrapolation of the  $K^*_{CO_2}$  values in a given hemoglobin solution to the point where  $(Hb^-) = 0$ , one obtains  $K'_{CO_2}$ . From Fig. 6 we get  $K'_{CO_2} = (6.4 \pm 0.5) \times 10^{-7}$ , in agreement with the value calculated from the ionic strength.

In Table X are shown the data of Margaria and Green (1933) calculated in the same way. The following points in this analysis are emphasized: (1) The correlation coefficient of  $K^*_{\text{CO}_2}$  and  $(\text{Hb}^-)$  is in all cases significant, showing, as expected, that they are in linear relation; (2)  $K_{\text{Am}} \times 10^6$  is  $3.8 \pm 0.5$  for horse hemoglobin at  $38^\circ$ , a value significantly different from but of the order of our value of  $6.4 \pm 0.5$  for beef hemoglobin; (3)  $K'_{\text{CO}_2}$  is in most cases not significantly different from the value calculated from the ionic strength. It appears reasonable to accept the conclusion that our experiments and those of Margaria and Green are in agreement with Equation 13; we regard this agreement as further proof of the mechanism of the carbamate equilibrium presented here.

$\frac{K_{\text{Am}}}{K'_{\text{CO}_2}}$  in *Reduced Red Blood Cell Contents*—Van Slyke, Hastings, Murray, and Sendroy (1925) hemolyzed serum-free horse red cells with saponin and equilibrated the solution of red cell contents with  $\text{CO}_2$ . Using Equation 11 in the form  $\text{p}K^*_{\text{CO}_2} = \text{pH} - \log \frac{(\overline{\text{CO}_2})}{\alpha_{\text{CO}_2} P_{\text{CO}_2}}$ , they found a mean value of  $\text{p}K^*_{\text{CO}_2}$  of 5.93 at  $38^\circ$  in eleven

determinations. Equation 13 in the form  $\frac{K^*_{\text{CO}_2}}{K'_{\text{CO}_2}} = 1 + \frac{K_{\text{Am}}}{K'_{\text{CO}_2}} (\text{Hb}^-)$  allows us to calculate  $K_{\text{Am}}/K'_{\text{CO}_2}$  in cell contents from this experiment. The mean value of  $(\text{Hb}^-)$  in their experiments was 0.04 M, and  $\text{p}K'_{\text{CO}_2}$  calculated from the ionic strength is 6.10; whence, by substitution of these values into the equation, we calculate that  $K_{\text{Am}}/K'_{\text{CO}_2} = 12$  and  $K_{\text{Am}} = 9.5 \times 10^{-6}$ . These values are in substantial agreement with those calculated from the direct determination of carbamate in beef hemoglobin (see Table VI).

*Non-Carbonate Equilibrium and Calculation of  $K_{\text{Am}}$  in Serum*—Siegfried (1905) using conductivity methods had early shown, in a paper which unfortunately was disregarded by most workers in the field, that serum protein forms carbamates. We have confirmed this by a study of the non-carbonate equilibrium of horse serum protein, and, in addition, we have calculated the carbamate equilibrium constant at  $0^\circ$  from our data.

According to the carbamate hypothesis, one should find the curve of serum protein carbamate against  $P_{\text{CO}_2}$  to have the same form at equilibrium, and the value of  $K_{\text{Am}}$  to have the same mag-



nitude as in the case of hemoglobin and the simple amino acids. This we found to be the case (see Fig. 7) in the following experiment. A serum protein solution was equilibrated at  $0^\circ$ , and the protein carbamate ( $\text{Prot} \cdot \text{Am}^-$ ) and  $P_{\text{CO}_2}$  at equilibrium were calculated as described in the case of the non-carbonate equilibrium of hemoglobin. Since the amount of carbamate forming in serum would be expected to be small, we increased it, in order to diminish the experimental error, by using a concentrated (30 per cent) solution of horse serum protein containing a high concentration of avail-

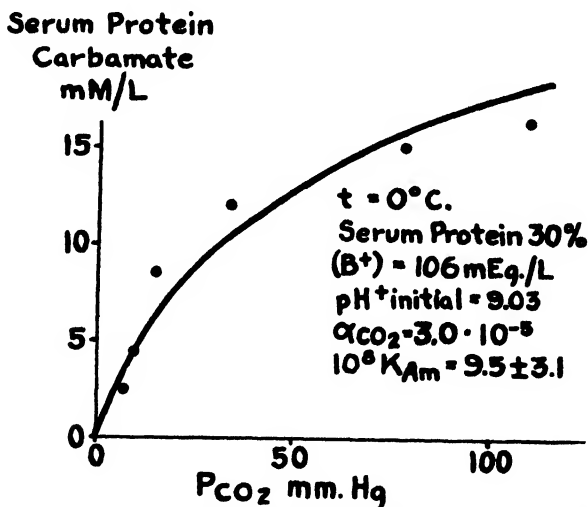


FIG. 7. The non-carbonate equilibrium of horse serum protein and  $\text{CO}_2$ . The full line is calculated from the equilibrium constant.

able base. A dried hydrophillic horse serum protein prepared by the method of Flosdorf and Mudd (1935) was excellently suited to this purpose. The available base in the solution was calculated by analysis of the total base and subtraction of the determined chlorides. The proteinate ( $\text{Prot}^-$ ) was calculated from  $(\text{B}^+)$  and  $(\text{Prot} \cdot \text{Am}^-)$  by an analogue of Equation 3-a; pH was calculated by an equation similar to Equation 1, viz.  $(\text{Prot}^-) = 0.11 (P)$  ( $\text{pH} - 5.48$ ). (Here  $(P)$  = gm. of protein per liter.) The constants of this equation were determined by titration of the concentrated protein solution with  $\text{HCl}$ , and they are in close agree-

ment with those found by Van Slyke, Wu, and McLean (1923) for normal horse serum protein. Using Equation 2 in the form

$$\frac{(\text{Prot} \cdot \text{Am}^-)}{(\text{Prot}^-)} = \frac{\alpha_{\text{CO}_2} P_{\text{CO}_2} K_{\text{Am}}}{(\text{H}^+)} \quad (14)$$

one can calculate, by the same method used in the case of hemoglobin, the value of  $K_{\text{Am}}$ . This was found to be  $(9.5 \pm 3.1) \times 10^{-8}$ —a value (at  $0^\circ$ ) one-third to one-fourth that of hemoglobin. This is not surprising, because amino acids may differ 10-fold in their values of  $K_{\text{Am}}$  (as do cysteic acid and glycine), and serum protein is weaker in amphanion formation than hemoglobin. The full line in Fig. 7 was calculated from this mean value of  $K_{\text{Am}}$ ; and the agreement of the experimentally determined points with this line shows, we believe, that the mass action equation applies in the case of serum proteins as well as in the case of hemoglobin. It is probable, we think, that carbamate formation is common to all proteins.

*Concentration of Carbamate in Normal Plasma at  $38^\circ$* —The value of  $K_{\text{Am}}$  of plasma at  $38^\circ$  can be calculated from its value at  $0^\circ$  by assuming that the heat of the carbamate reaction is the same as in the case of hemoglobin; i.e., 12,000 calories. The equation of van't Hoff gives  $K_{\text{Am}} = (6.4 \pm 2.1) \times 10^{-7}$  at  $38^\circ$ . In normal plasma at pH = 7.4 and  $P_{\text{CO}_2} = 40$  mm. of Hg,  $(\text{Prot}^-)$  is about 0.016 M. By Equation 14 we calculate (using  $\alpha_{\text{CO}_2} = 3.1 \times 10^{-8}$ ) the concentration of plasma carbamate to be  $0.5 \pm 0.2$  mM per liter. This calculation indicates that small amounts of carbamate may be present in normal plasma, but the amount is less than the present methods of analysis can demonstrate with certainty.

*Mechanisms in Rapid Uptake of  $\text{CO}_2$  by Plasma*—The plasma of the blood in its passage through the tissue capillaries increases its load of  $\text{CO}_2$  by about 5 volumes per cent, but, the approximate time of capillary passage being 1 second, this loading of  $\text{CO}_2$  must be accomplished rapidly. A consideration of the time factor of loading reveals two new mechanisms in  $\text{CO}_2$  transport. Formerly, it was believed that the capillary plasma could take up  $\text{CO}_2$  with sufficient rapidity (1) by a small increase of free  $\text{CO}_2$ , due to the increase of  $P_{\text{CO}_2}$  from 40 to 45 mm. of Hg, and (2) by a large increase of bicarbonate, due to the hydration of  $\text{CO}_2$  to  $\text{H}_2\text{CO}_3$  in the plasma. Henriques (1928), however, showed that  $\text{CO}_2$  hydra-

tion in separated plasma is so slow that only about one-twentieth of the total loading in 1 second by capillary plasma could be accounted for by this mechanism. This, as is now known, is because carbonic anhydrase—the enzyme accelerating hydration enormously—is entirely absent from the plasma though abundant in the red cells. Mechanisms other than hydration in the plasma must be operating, and, since the direct combination of  $\text{CO}_2$  with plasma proteins as carbamate is rapid, its rôle in the rapid uptake of  $\text{CO}_2$  must be evaluated. From Equation 14, however, using the calculated value of  $K_{Am}$  at  $38^\circ$ , we calculate that the increase of plasma carbamate, owing to the increase of  $P_{\text{CO}_2}$  from 40 to 45 mm. of Hg, is only 0.06 volume per cent—an amount equal to only one-fourth of the additional free  $\text{CO}_2$ . In other words, considerable  $\text{CO}_2$  must be rapidly loaded into the plasma by still another mechanism than physical solution, carbamate formation, or uncatalyzed hydration in the plasma. Roughton (1935) has suggested that the capillary plasma acquires the remaining  $\text{CO}_2$  as bicarbonate in the following way: Free  $\text{CO}_2$  from the tissues migrates rapidly through the plasma into the red cells, where, since carbonic anhydrase is abundant, it is practically instantaneously hydrated to  $\text{H}_2\text{CO}_3$ . This, in turn, forms bicarbonate ions. The bicarbonate then rapidly passes into the plasma, but it can only do so by exchanging with an equivalent amount of chloride ions. The approximate rôles of these four mechanisms in the rapid uptake of  $\text{CO}_2$  by the plasma during the 1 second of capillary passage are as follows:

| $\text{CO}_2$ rapidly taken up                                     | vol. per cent |
|--|---------------|
| As free $\text{CO}_2$ .....  | 0.4           |
| “ carbamate.....   | 0.1           |
| By hydration to $\text{H}_2\text{CO}_3$ in plasma.....             | 0.3           |
| From cells as $\text{HCO}_3^-$ in exchange for $\text{Cl}^-$ ..... | 4.2           |

It appears, then, that the very rapid hydration of  $\text{CO}_2$  to  $\text{H}_2\text{CO}_3$ , catalyzed by carbonic anhydrase *within the red cell*, together with the rapid exchange of plasma chloride for cell bicarbonate, accounts for 80 per cent of the rapid uptake of  $\text{CO}_2$  by the *plasma*. This ingenious hypothesis of Roughton, which seems inescapable, is a most unexpected and unique development of the subject of  $\text{CO}_2$  transport.

**Partition of  $\text{CO}_2$  in Plasma and Cells of Whole Blood**—It is not necessary to discuss this subject in detail since this has been adequately done by Roughton (1935). It will be shown, however, that the calculation of the partition of the total bound  $\text{CO}_2$  between

TABLE XI

*Partition of Carbon Dioxide in Plasma and Cells of Whole Blood of A.V.B. at 38°; Oxygen Capacity 20.0 Volumes Per Cent*

|   | Arterial |       |             | Venous |       |             | Difference (increase) |       |             |
|---|----------|-------|-------------|--------|-------|-------------|-----------------------|-------|-------------|
|   | Plasma   | Cells | Whole blood | Plasma | Cells | Whole blood | Plasma                | Cells | Whole blood |
| Hematocrit . . . . .                                  | 0.600    | 0.400 |             | 0.596  | 0.404 |             |                       |       |             |
| Oxygen saturation, %..                                |          | 96    |             |        | 74    |             |                       |       |             |
| $K_{\text{Am}}/K'_{\text{CO}_2}$ . . . . .            | 2.3      | 3.6   |             | 2.3    | 4.8   |             |                       |       |             |
| Available base, m.-eq. per l. blood . . . . .         | 23.1     | 27.8  | 51.0        | 24.0   | 27.0  | 51.0        |                       |       |             |
| Proteinate " " . . . . .                              | 7.9      | 22.6  | 30.5        | 7.8    | 21.2  | 30.0        |                       |       |             |
| pH . . . . .  | 7.45     | 7.12  |             | 7.43   | 7.11  |             |                       |       |             |
| $P_{\text{CO}_2}$ , mm. Hg . . . . .                  | 40.0     | 40.0  | 40.0        | 45.4   | 45.4  | 45.4        |                       |       |             |
| Free $\text{CO}_2$ , cc. per 100 cc. blood . . . . .  | 1.6      | 0.8   | 2.4         | 1.8    | 0.9   | 2.7         | 0.2                   | 0.1   | 0.3         |
| Bound $\text{CO}_2$ , cc. per 100 cc. blood . . . . . | 34.1     | 11.8  | 35.9        | 36.3   | 13.1  | 49.4        | 2.2                   | 1.3   | 3.5         |
| Bicarbonate, cc. per 100 cc. blood . . . . .          | 33.1     | 9.8   | 42.9        | 35.2   | 10.5  | 45.6        | 2.1                   | 0.7   | 2.8         |
| Carbamate, cc. per 100 cc. blood . . . . .            | 1.0      | 2.0   | 3.0         | 1.1    | 2.6   | 3.7         | 0.04                  | 0.6   | 0.7         |
| Total $\text{CO}_2$ , cc. per 100 cc. blood . . . . . | 35.7     | 12.5  | 38.2        | 38.0   | 14.0  | 52.0        | 2.4                   | 1.4   | 3.8         |
| Carbamate to total $\text{CO}_2$ , % . . . . .        | 2.8      | 16    | 8           | 2.8    | 19    | 7           | 1.7                   | 45    | 18          |

carbamate and bicarbonate can be most simply made by means of the mass action relations developed in this paper; viz.:

The bound carbon dioxide is  $(\overline{\text{CO}_2}) = (\text{HCO}_3^-) + (\text{HbAm}^-)$ ; further  $\alpha_{\text{CO}_2} P_{\text{CO}_2} = (\text{HCO}_3^-)(\text{H}^+)/K'_{\text{CO}_2}$ ; therefore, Equation 2 becomes

$$\frac{(\text{HbAm}^-)}{(\overline{\text{CO}_2})} = \frac{1}{1 + \frac{K_{\text{CO}_2}/K_{\text{Am}}}{(\text{Hb}^-)}} \quad (15)$$

This equation gives the proportion of the total bound  $\text{CO}_2$  which exists in a hemoglobin solution as carbamate. (Here  $(\text{Hb}^-)$  must be expressed in equivalents per liter.) The bicarbonate, of course, can be calculated from the carbamate and total bound  $\text{CO}_2$  by difference.

It is assumed, in lieu of other data, that the values of  $K_{\text{Am}}/K'_{\text{CO}_2}$ , determined for horse reduced and oxyhemoglobin at  $38^\circ$ , and calculated in the case of serum from its value at  $0^\circ$ , are the same for human blood. It is further assumed that  $K_{\text{Am}}/K'_{\text{CO}_2}$  varies proportionately to the degree of oxygenation of hemoglobin; its value in venous cells is calculated accordingly. The calculations for the blood of A.V.B. (Henderson, 1928) are given in Table XI.<sup>7</sup> They show (1) a small percentage (3 per cent) of the total  $\text{CO}_2$  as carbamate in the arterial plasma, not increasing in venous plasma; (2) 16 per cent of the arterial cell  $\text{CO}_2$  as carbamate, increasing to 19 per cent in the venous cells; (3) of the total arterio-venous exchange of  $\text{CO}_2$ , 45 per cent in the cells and 18 per cent in the whole blood are due to carbamate. These calculations are in substantial agreement with those of Roughton.

It may again be emphasized (see Fig. 5) that the curves of carbamate in physiological ranges of  $P_{\text{CO}_2}$  and pH are virtually horizontal and that the reduced hemoglobin curve is at a higher level than the oxyhemoglobin curve. This means that the increase of carbamate in the venous blood is due solely to the greater ability of reduced hemoglobin to form carbamate. The oxylabile group of the hemoglobin molecule, in which this increased "affinity" for carbamate undoubtedly resides, is thus recognized as possessing further significance in blood gas transport besides that of carrying oxygen; namely, the transport of  $\text{CO}_2$  as carbamate.

#### SUMMARY

1. Old and new evidence is given showing that the carbamate hypothesis—namely, that  $\text{CO}_2$  combines directly with the  $-\text{NH}_2$  groups of the protein amphanion (proteinate) and not with the zwitter ion (isoelectric protein)—is applicable in the case of hemoglobin.

2. All or many of the  $-\text{NH}_2$  groups of the protein are con-

<sup>7</sup> The proteinate concentration must be in equivalents per liter of cells in Equation 15, not milli-equivalents per liter of blood as in Table XI.

cerned in the combination of  $\text{CO}_2$  as carbamate rather than a single group of the oxygen linkage.

3. The combination is a carbamate ( $\text{HbAm}^-$ ) binding 1 equivalent of base per mole of  $\text{CO}_2$ .

4. The distinction between the total and the non-carbonate equilibrium is given, the mass action law is applied to both, and carbamate equilibrium constants are experimentally calculated.

5. The difference of carbamate concentration in reduced and oxyhemoglobin can be calculated from the ionization constants of the oxylabile group. Experiments confirm the calculations.

6. The heat of the carbamate reaction is calculated from the equilibrium constants at two temperatures.

7. The relation of the bicarbonate pseudoconstant,  $K^*_{\text{CO}_2}$ , to the carbamate constant,  $K_{\text{Am}}$ , and the bicarbonate constant,  $K'_{\text{CO}_2}$ , is deduced and shown to agree with experiment.

8. Serum proteins are shown to form carbamate. The carbamate mass action constant for serum protein is calculated from experimental data, and the concentration of carbamate in physiological plasma estimated. The rôles of four mechanisms in the rapid uptake of  $\text{CO}_2$  by the capillary plasma are evaluated.

9. The equation for the proportion of total bound  $\text{CO}_2$  as carbamate in protein solutions is deduced. By it the partition of the total  $\text{CO}_2$  as bicarbonate and carbamate in the plasma and the cells of whole blood is calculated, and the rôle of carbamate in the transport of  $\text{CO}_2$  estimated.

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# ON SOME CHANGES IN THE CHEMICAL COMPOSITION OF THE BLOOD OF THE TURTLE FOLLOW- ING COMPLETE ANOXIA\*

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It was shown in a previous paper by Johlin and Moreland (1) that the turtle is extremely resistant to complete anoxia, being able to survive under such conditions for 30 hours or longer. At the end of such a period the fermentable blood sugar of the animal was found to have increased from a normal of 50 mg. per cent to as much as 1100 mg. per cent, the lactic acid of the blood to have increased to as much as 1000 mg. per cent, and its pH to have dropped from 7.9 to 6.8 with an accompanying loss of carbonate greater than that contained in all body fluids combined. Prolonged periods of anoxia resulting in such extreme changes in the blood picture of an animal had not been studied before.

The present paper contains a more detailed study of the inter-relationship of the changes mentioned above in the blood picture, and the influence of various factors which might bring about changes in the blood picture and might affect the animal's resistance to anoxia, such as the administration of alkali, cyanide, and iodoacetate.

All of the animals used in the present experiments were fresh water turtles, mostly Blandings (*Emys blandingii*) and painted turtles (*Chrysemys picta*, *Chrysemys marginata*, *Chrysemys belli*). While painted turtles were found to be somewhat more resistant to asphyxia than others which were tried, there appears to be no difference in the reactions of different species of painted turtles.

\* Taken from a thesis presented to the faculty of Vanderbilt University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, January, 1936.



Most of the experiments were carried out in a constant temperature laboratory at 20°. Experiments involving anoxic anoxia were carried out with a large vacuum desiccator as described previously. Histotoxic anoxia was produced by injecting a 10 per cent solution of sodium cyanide intraperitoneally. The dosage was usually 100 mg. per kilo of body weight. Blood was taken from the animals as described previously except that in the experiments involving histotoxic anoxia, blood was drawn from the carotid artery by means of an oiled syringe. In the case of some moribund animals blood was drawn from the jugular vein or from the heart. Alkali, when administered, was injected intraperitoneally by drilling a hole through the plastron on the mid-line and slightly caudad of center, and inserting the stub of a hypodermic needle to which was attached a rubber tube leading outside of the asphyxiation chamber. Determinations of pH, blood sugar, lactic acid, and CO<sub>2</sub> were made as previously described. In a few cases tests were made for the presence of methylglyoxal and pyruvic acid, for the former by the method of Barrenscheen and Braun (2), for the latter by that of Simon and Piaux (3) as described by Case and Cook (4).

The main data obtained in these experiments are contained in Table I. All experiments here noted were carried out under conditions of complete oxygen lack except in the case of cyanide administration, where it was assumed that the inhibition of respiration by blocking the respiratory enzymes produced an anoxia equivalent to that of a total lack of oxygen in the respiratory chamber. The average accumulation of lactate and fermentable sugar in the blood, as well as the average lowering of pH, was greater under conditions of cyanide administration than under those of a complete lack of oxygen. Under both types of anoxia it is to be observed that the administration of base increases the formation of lactate and reduces the amount of fermentable sugar, greatly reducing the ratio of sugar to lactate. This course of events was predicted on the assumption that a diminution of acidosis would allow a larger proportion of glucose to be converted into lactic acid since this change is accompanied by a far greater liberation of energy than the transformation of glycogen into glucose. The fact that the pH of the blood under alkali administration is lower than normal, while the total CO<sub>2</sub> content is higher,

TABLE I  
*Chemical Composition of Blood of Turtles under Various Conditions*

| Turtle No. | Treatment  | Duration of anoxia | Solution injected per kilo body weight | NaHCO <sub>3</sub> per kilo equivalent to lactic acid formed | Glucose fed per kilo | pH   | CO <sub>2</sub> | Sugar        | Lactic acid  | Ratio of sugar to lactic acid |
|------------|--|--------------------|--|--|----------------------|------|-----------------|--------------|--------------|-------------------------------|
|            |  | hrs.               | cc.                                    | cc.  | gm.                  |      | vol. per cent   | mg. per cent | mg. per cent |                               |
| 114        | Normal   |                    |  |  |                      | 7.60 | 94              | 43           | 36           |                               |
| 136        | "  |                    |  |  |                      | 7.83 |                 | 56           | 23           |                               |
| 140        | "  |                    |  |  |                      | 7.72 | 76              | 37           | 18           |                               |
| 144        | "  |                    |  |  |                      | 7.83 |                 | 48           | 22           |                               |
| 149        | "  |                    |  |  |                      | 7.81 | 91              | 38           | 33           |                               |
| 156        | "  |                    |  |  |                      | 7.72 | 84              | 235          | 19           |                               |
| 168        | "  |                    |  |  |                      | 7.75 | 72              | 16           | 10           |                               |
| 169        | "  |                    |  |  |                      | 7.63 | 69              | 25           | 29           |                               |
| 181        | "  |                    |  |  | 5                    | 7.62 | 81              | 50           | 20           |                               |
| 186        | "  |                    |  |  | 5                    | 7.66 | 65              | 50           | 19           |                               |
| 192        | "  |                    |  |  | 5                    | 7.74 | 88              | 116          | 21           |                               |
| 194        | "  |                    |  |  | 5                    | 7.68 | 84              | 57           | 45           |                               |
| 195        | "  |                    |  |  | 5                    | 7.98 | 63              | 34           | 40           |                               |
| 196        | "  |                    |  |  |                      | 7.94 | 68              | 40           | 61           |                               |
| 207        | "  |                    |  |  |                      | 7.79 | 92              | 33           | 9            |                               |
| 135        | N <sub>2</sub> + Na <sub>2</sub> CO <sub>3</sub> | 26                 | 16.5                                   |  |                      | 7.1  |                 | <50          | 786          | 0.05                          |
| 138        | " + "  | 30                 | 37                                     |  |                      | 7.50 |                 | 20           | 957          | 0.02                          |
| 139        | " + "  | 22                 | 33                                     |  |                      | 7.22 |                 | 88           | 852          | 0.10                          |
| 142        | " + "  | 24                 | 33                                     |  |                      | 7.13 |                 | 35           | 928          | 0.04                          |
| 151        | " + "  | 27                 | 33                                     |  |                      | 7.48 |                 | 334          | 1135         | 0.29                          |
| 154        | " + "  | 24                 | 33                                     |  |                      | 7.54 | 63              | 282          | 975          | 0.29                          |
| 184        | " + "  | 23                 | 33                                     |  | 5                    | 7.30 | 49              | 120          | 1065         | 0.11                          |
| 190        | " + "  | 27                 | 33                                     |  | 5                    | 7.31 | 131             | 366          | 1150         | 0.32                          |
| 191        | " + "  | 24                 | 33                                     |  | 5                    | 7.39 | 92              | 314          | 1105         | 0.28                          |
| 115        | " + NaHCO <sub>3</sub>                           | 23                 | 26.2                                   | 35   |                      | 7.30 | 47              | 200          | 677          | 0.30                          |
| 116        | " + "  | 23                 | 34.8                                   | 42   |                      | 7.25 | 39              | 514          | 830          | 0.62                          |
| 121        | " + "  | 24                 | 30.0                                   | 38   |                      | 7.18 | 54              | 452          | 740          | 0.61                          |
| 122        | " + "  | 23                 | 27.0                                   | 46   |                      | 7.20 | 32              | 342          | 900          | 0.38                          |
| 124        | " + "  | 25                 | 34.5                                   | 47   |                      | 7.43 | 20              | 181          | 925          | 0.20                          |

TABLE I—Continued

| Turtle No. | Treatment                              | Duration of anoxia |      | Solution injected per kilo body weight | NaHCO <sub>3</sub> per kilo equivalent to lactic acid formed | Glucose fed per kilo | pH   | CO <sub>2</sub> | Sugar | Lactic acid | Ratio of sugar to lactic acid |
|------------|--|--------------------|------|--|--|----------------------|------|-----------------|-------|-------------|-------------------------------|
|            |  | hrs.               | cc.  |  |  | gm.                  |      |                 |       |             |                               |
| 143        | N <sub>2</sub> + NaCl                  | 24                 | 33.5 |  |  |                      | 6.86 | 18              | 114   | 361         | 0.32                          |
| 145        | " + "                                  | 24                 | 33   |  |  |                      | 6.92 | 23              | 313   | 417         | 0.75                          |
| 148        | " + "                                  | 25                 | 33   |  |  |                      | 6.80 |                 | 558   | 500         | 1.12                          |
| 150        | " + "                                  | 23                 | 33   |  |  |                      | 7.07 | 16              | 254   | 472         | 0.54                          |
| 155        | " + "                                  | 24                 | 33   |  |  |                      | 7.00 |                 | 178   | 401         | 0.44                          |
| 166        | " + "                                  | 23                 | 33   |  |  |                      | 6.68 | 20              | 145   | 425         | 0.34                          |
| 185        | " + "                                  | 23                 | 31.5 |  |  | 5                    | 6.75 | 27              | 365   | 435         | 0.84                          |
| 187        | " + "                                  | 27                 | 33   |  |  | 5                    | 6.72 | 19              | 580   | 557         | 1.04                          |
| 189        | " + "                                  | 25                 | 33   |  |  | 5                    | 6.86 | 21              | 1010  | 535         | 1.89                          |
| 117        | "                                      | 26                 |      |  |  |                      | 7.12 | 34              | 545   | 525         | 1.04                          |
| 118        | "                                      | 25                 |      |  |  |                      | 7.10 | 13              | 590   | 580         | 1.02                          |
| 119        | "                                      | 25                 |      |  |  |                      | 7.05 | 36              | 500   | 568         | 0.88                          |
| 141        | "                                      | 24                 |      |  |  |                      | 6.83 | 11              | 416   | 613         | 0.68                          |
| 146        | "                                      | 25                 |      |  |  |                      | 6.95 | 19              | 230   | 665         | 0.35                          |
| 157        | "                                      | 25                 |      |  |  |                      | 6.92 | 21              | 360   | 648         | 0.56                          |
| 161        | "                                      | 26                 |      |  |  |                      | 6.88 | 17              | 354   | 681         | 0.52                          |
| 162        | "                                      | 24                 |      |  |  |                      | 6.97 | 21              | 23    | 722         | 0.03                          |
| 182        | "                                      | 27                 |      |  |  | 5                    | 6.97 | 27              | 145   | 691         | 0.21                          |
| 188        | "                                      | 24                 |      |  |  | 5                    | 6.83 | 28              | 992   | 594         | 1.67                          |
| 193        | "                                      | 24                 |      |  |  | 5                    | 6.89 | 28              | 596   | 600         | 0.99                          |
| 197        | "                                      | 25                 |      |  |  | 5                    | 6.86 | 19              | 836   | 663         | 1.26                          |
| 198        | "                                      | 22                 |      |  |  | 5                    | 6.89 | 33              | 696   | 645         | 1.08                          |
|            |  |                    |      |  | NaCN per kilo  |                      |      |                 |       |             |                               |
|            |  |                    |      |  | mg.  |                      |      |                 |       |             |                               |
| 210        | NaCN + Na <sub>2</sub> CO <sub>3</sub> | 21                 | 33   |  | 100  |                      |      |                 | 352   |             |                               |
| 212        | " + "                                  | 23                 | 33   |  | 100  |                      |      |                 | 177   |             |                               |
| 213        | " + "                                  | 22                 | 33   |  | 100  |                      |      |                 | 117   | 800         | 0.15                          |
| 223        | " + "                                  | 22                 | 33   |  | 100  |                      |      |                 | 629   | 741         | 0.85                          |
| 229        | " + "                                  | 20                 | 33   |  | 100  |                      | 7.09 | 85              | 430   | 960         | 0.45                          |
| 232        | " + "                                  | 20                 | 33   |  | 100  |                      |      |                 | 306   | 746         | 0.41                          |
| 233        | " + "                                  | 20                 | 33   |  | 100  |                      | 7.33 | 89              | 358   | 721         | 0.50                          |
| 227        | " + "                                  | 19                 | 33   |  | 20   |                      |      |                 | 188   | 882         | 0.21                          |
| 228        | " + "                                  | 20                 | 33   |  | 20   |                      | 7.31 | 102             | 339   | 985         | 0.36                          |

TABLE I—*Concluded*

| Turtle No. | Treatment | Duration of anoxia | Solution injected per kilo body weight | NaCN per kilo | Glucose fed per kilo | pH    | CO <sub>2</sub> | Sugar        | Lactic acid  | Ratio of sugar to lactic acid |
|------------|-----------|--------------------|--|---------------|----------------------|-------|-----------------|--------------|--------------|-------------------------------|
|            |           | hrs.               | cc.                                    | mg.           | gm.                  |       | vol. per cent   | mg. per cent | mg. per cent |                               |
| 204        | NaCN      | 22                 |  | 100           |                      | 6.72  | 17              | 720          | 670          | 1.07                          |
| 205        | "         | 22                 |  | 100           |                      | 6.97  |                 | 792          | 670          | 1.18                          |
| 206        | "         | 22                 |  | 100           |                      | 6.76  | 23              | 812          | 725          | 1.12                          |
| 208        | "         | 18                 |  | 100           |                      | 6.72  | 23              | 480          | 650          | 0.74                          |
| 209        | "         | 22                 |  | 100           |                      | 6.72  | 17              | 852          | 740          | 1.15                          |
| 214        | "         | 20                 |  | 100           |                      | 6.63  | 33              | 512          | 571          | 0.90                          |
| 222        | "         | 19                 |  | 50            |                      | 6.72  | 28              | 526          | 593          | 0.89                          |
| 226        | "         | 22                 |  | 20            |                      | 7.44* | 23              | 600          | 555          | 1.08                          |
| 230        | "         | 21                 |  | 20            |                      | 7.31* | 13              | 642          | 540          | 1.19                          |

## Averages

|  |  |  |  |  |      |    |     |     |      |
|--|--|--|--|--|------|----|-----|-----|------|
| Normal   |  |  |  |  | 7.75 | 79 | 59  | 27  |      |
| N <sub>2</sub> + Na <sub>2</sub> CO <sub>3</sub> |  |  |  |  | 7.33 | 84 | 177 | 995 | 0.17 |
| " + NaHCO <sub>3</sub>                           |  |  |  |  | 7.27 | 38 | 338 | 814 | 0.42 |
| " + NaCl   |  |  |  |  | 6.85 | 21 | 391 | 467 | 0.81 |
| "  |  |  |  |  | 6.94 | 24 | 483 | 630 | 0.79 |
| NaCN + Na <sub>2</sub> CO <sub>3</sub>           |  |  |  |  | 7.24 | 92 | 322 | 826 | 0.42 |
| "  |  |  |  |  | 6.75 | 22 | 659 | 635 | 1.04 |

\* Not included in average.

must be attributed to retention of excessive amounts of free carbonic acid at the moribund stage attained by the animal after some 20 hours of complete anoxia.

The relative amount of blood sugar existing as fermentable sugar in the blood of these animals is shown in Table II. That the hyperglycemia of anoxia is not entirely due to the attendant acidosis was illustrated by allowing animals to breathe high concentrations of CO<sub>2</sub> in the presence of oxygen, as shown by the data of Table III. The occasional rather high blood sugar of a normal animal and the occasional low blood sugar following anoxia cannot be explained at present.

As shown by Johlin and Moreland (1) the amount of CO<sub>2</sub>

expired during anoxia is greater than that expired normally by the same animal. Further experiments were carried out to compare the amount of expired  $\text{CO}_2$  presumably set free by the lactic acid formed during anoxia with the amount of lactate accumulated during this period. To make this comparison it was assumed that lactate is distributed uniformly through all soft tissues, that the

TABLE II  
*Saccharoid Content of Blood of Normal and Asphyxiated Turtles*

| Turtle No. | Treatment              | Glucose +<br>saccharoids as<br>glucose | Saccharoids as<br>glucose |
|------------|------------------------|--|---------------------------|
|            |                        | mg. per cent                           | mg. per cent              |
| 2-a        | $\text{N}_2$ , 27 hrs. | 922                                    | 32                        |
| 4-a        | Normal                 | 263                                    | 0                         |
| 7-a        | "                      | 216                                    | <5                        |
| 12-a       | $\text{N}_2$ , 28 hrs. | 550                                    | 11                        |
| 13-a       | " 27 "                 | 575                                    | 5                         |
| 161        | " 26 "                 | 354                                    | 1                         |

TABLE III  
*Relative Effects of Anoxia and of Carbon Dioxide on Composition of Blood*

| Turtle No. | Gas                        | pH   | $\text{CO}_2$ | Sugar        | Lactic acid  |
|------------|----------------------------|------|---------------|--------------|--------------|
|            |                            |      | vol. per cent | mg. per cent | mg. per cent |
| 65         | $\text{N}_2$               | 7.23 | 20            | 1000         | 140          |
| 66         | "                          | 7.02 | 29            | 85           | 100          |
| 67         | "                          | 7.18 | 22            | 625          | 100          |
| 72         | $\text{O}_2 + \text{CO}_2$ | 7.15 | 175           | 93           | 6            |
| 73         | " + "                      | 7.09 | 132           | 290          | 12           |
| 74         | " + "                      | 7.22 | 167           | 195          | 14           |
| Average... | $\text{N}_2$               | 7.14 | 24            | 570          | 113          |
| " ...      | $\text{O}_2 + \text{CO}_2$ | 7.15 | 158           | 195          | 11           |

blood is one-seventh of the total of the soft tissues, as shown by Irving, Ferguson, and Plewes (5), and that the blood volume of the turtle is 80 cc. per kilo of body weight, as found by Derrickson (6). A comparison made on this basis is given in Table IV.

A number of experiments were carried out to determine whether insulin might affect the transformation of glycogen into glucose, and *vice versa*, under conditions of anoxia. It had been found

that turtles do not show an immediate response to insulin treatment but that they will exhibit hypoglycemia after a number of hours (7). Data obtained in the present experiments, as illustrated by Table V, show that there is a marked hyperglycemia  $4\frac{1}{2}$  hours after the administration of 25 units of insulin per kilo of body weight and that the hyperglycemia of prolonged anoxia is not reduced by insulin.

TABLE IV  
*Comparison of Carbon Dioxide Excretion with Lactic Acid Formation in Anoxia*

| Turtle No.....                | 116  | 117  | 118  | 119  | 122  |
|-------------------------------|------|------|------|------|------|
| CO <sub>2</sub> lost, mm..... | 50.0 | 41.6 | 26.6 | 24.9 | 48.9 |
| Lactic acid increase, mm..... | 43.2 | 36.0 | 23.7 | 24.3 | 48.5 |

TABLE V  
*Effect of Insulin on Blood Sugar of Turtles*

| Turtle No. | Treatment                              | Insulin          |                                     | Blood sugar          |               |
|------------|--|------------------|-------------------------------------|----------------------|---------------|
|            |  | Dose             | Time between injection and bleeding | At insulin injection | After insulin |
|            |  | units            | hrs.                                | mg. per cent         | mg. per cent  |
| 10-b       | Air                                    | 25               | 3                                   |                      | 45            |
| 11-b       | "                                      | 25               | 3                                   |                      | 64            |
| 13-b       | "                                      | 25               | $4\frac{1}{2}$                      | 37                   | 115           |
| 14-b       | "                                      | 25               | $4\frac{1}{2}$                      |                      | 265           |
| 16-b       | "                                      | 25               | $4\frac{1}{2}$                      | 43                   | 170           |
| 12-b       | N <sub>2</sub> , 25 $\frac{1}{2}$ hrs. | 25               | 2                                   |                      | 1080          |
| 15-b       | " 31 $\frac{1}{2}$ "                   | 15               | $5\frac{1}{2}$                      | 1070                 | 1170          |
| 239        | NaCN, 100 mg. per kilo 19 hrs.         | 50<br>(per kilo) | 120                                 |                      | 516           |

Sublethal or lethal doses of iodoacetate (pH 7.4 to 7.6) did not prevent the accumulation of lactates in the blood during anoxia even when administered 2 to 4 hours before anoxia was induced.

Analyses were made of the glycogen content of striped muscle, liver, and ventricles of some forty animals, both normal and asphyxiated, to determine, if possible, the participation of these various tissues in furnishing glycogen for the anaerobic metabolism

of these animals. The glycogen content of different animals was found to vary so greatly that the average results obtained from a limited number of cases could not be depended on. The feeding of glucose for a number of days previous to an experiment did not help to avoid such variations. It appeared, however, that anoxia accompanied by the administration of sodium carbonate diminished the liver glycogen less and the muscle glycogen more than in the case of untreated anoxia. It was also found that, while very considerable stores of glycogen remained in the muscle and liver of animals after prolonged anoxia, the glycogen of the ventricles disappeared almost completely.

#### SUMMARY

The previously reported high blood sugar and lactate produced by anoxia in the turtle can be modified by the administration of alkali during anoxia so as to lower the sugar content and further increase the lactate, thus greatly reducing the sugar to lactate ratio. The plasma pH and  $\text{CO}_2$  content do not fall so far below normal as when no alkali is given.

The severe acidosis produced by rebreathing  $\text{CO}_2$  in the presence of oxygen does not produce a hyperglycemia as does anoxia accompanied by a similar acidosis.

Practically all of the blood sugar produced in anoxia is fermentable.

Of the possible intermediate products of metabolism, methylglyoxal and pyruvic acid do not occur in significant amounts.

The amount of  $\text{CO}_2$  excreted during anoxia corresponds closely with the lactate formed.

Insulin will not lower the high sugar concentration of anoxia and iodoacetate will not prevent the accumulation of lactate.

Cardiac glycogen is almost entirely depleted during anoxia, while that of skeletal muscle is consumed but partially. The administration of alkali during anoxia apparently causes liver glycogen to be consumed to a lesser extent than in untreated anoxia.

The writer wishes to express his appreciation to Dr. J. M. Johlin, under whose direction this work was done, for much advice and help given during the course of the investigation, and to Dr. C. S.

Robinson for his interest and for making it possible for the work to be done.

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## A METHOD FOR THE QUICK DRY ASHING OF BLOOD PLASMA AND WHOLE BLOOD FOR THE DETERMINATION OF CHLORIDES

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In making a great many chloride determinations it is quite desirable to have a method which will give reliable results and also require the minimum amount of time for the procedure. Several methods are available for the determination of chlorides in plasma and whole blood. The most desirable seems to be the principle of Volhard as applied by Van Slyke (1) and Eisenman (2).

The procedure outlined here and the solutions used are similar to those described by Van Slyke (1) with the exception that the sample, instead of being wet-ashed, is dry-ashed with  $\text{AgNO}_3 \cdot \text{HNO}_3$  and magnesium nitrate, and requires only about 3 minutes for the operation.

*Reagents*—The  $\text{AgNO}_3 \cdot \text{HNO}_3$ , sulfocyanate, and ferric alum solutions are the same as those described by Van Slyke (1).

*Magnesium nitrate solution.* Make a warm saturated solution of the salt, allow it to come to room temperature, and filter.

The  $\text{AgNO}_3 \cdot \text{Mg}(\text{NO}_3)_2 \cdot \text{HNO}_3$  solution for use in the alternative procedure. The  $\text{AgNO}_3 \cdot \text{HNO}_3$  and  $\text{Mg}(\text{NO}_3)_2$  solutions may be mixed in the exact proportions of 3:2 and used as one solution. This is for convenience and requires only one transfer of reagent.

*Chloride-free asbestos.* To prepare enough asbestos for approximately 100 determinations, extract 5 gm. of medium or fine fiber acid-washed asbestos with about 200 cc. of distilled water to which about 15 cc. of nitric acid have been added. Allow to stand 2 or 3 hours with occasional vigorous shaking. Filter by any suitable means, wash several times with distilled water, and dry in an oven. When it is completely dry, break up the matted asbestos into a fluffy state.

*Procedure*

The blood is oxalated as in other procedures. Deliver 1 cc. of plasma or whole blood into a tall (4.5 by 7.5 cm.) 100 cc. silica beaker, add slowly and with constant stirring (shaking the beaker) exactly 3 cc. of  $\text{AgNO}_3 \cdot \text{HNO}_3$  reagent from a microburette, mix thoroughly by gently rotating the beaker, add 2 cc. of the saturated magnesium nitrate solution, and again mix in the same way. Add approximately 50 mg. of finely divided fluffy asbestos fiber. Mix again by rotating the beaker. Place on a triangle or plain wire gauze and heat gently at first and then over the full flame of a Bunsen burner. The heating should be stopped when the brown nitrous oxide fumes cease to come off. A white or gray ash is obtained. This requires about 2 to 3 minutes under our working conditions. Allow the beaker to cool, moisten the ash with about 3 cc. of water from a wash bottle, delivering it onto the sides of the beaker, and add slowly 5 cc. of concentrated nitric acid, using it to wash down the sides of the beaker. Shake the beaker gently to aid solution of the ash, heat gently for about 1 minute, cool thoroughly by placing in a pan of cool tap water, add 10 cc. of distilled water, and proceed immediately with indicator and titration as described by Van Slyke (1). The ash should not be taken up in solution with the nitric acid and water until ready for titration. The calculation is the same as that given by Van Slyke. The chloride values obtained by using this and other methods are given in Table I. Good results were also obtained when 0.5 cc. samples were used.

*Alternative Procedure*—The magnesium nitrate can be incorporated with the  $\text{AgNO}_3 \cdot \text{HNO}_3$  solution. If this is done, use 5 cc. of the  $\text{AgNO}_3 \cdot \text{Mg}(\text{NO}_3)_2 \cdot \text{HNO}_3$  reagent (instead of the  $\text{AgNO}_3 \cdot \text{HNO}_3$  and  $\text{Mg}(\text{NO}_3)_2$  as given in the first procedure) and ash in the same way. In this procedure the oxidation is more vigorous and less intense heating is necessary.

The ferric alum indicator can be mixed with the nitric acid used to dissolve the ash, but this imparts a slightly yellow color to the mixture, which is not present when they are added separately. In the standardization of the sulfocyanate the titrations are carried out in the presence of approximately the amount of asbestos and the exact amount of  $\text{Mg}(\text{NO}_3)_2$  solution used in ashing

the samples. This, of course, serves as a blank on these substances.

By this ashing technique several samples may be ashed at one

TABLE I  
*Chloride in Blood and Plasma*

| Method   | Cl per 100 cc. |        |       |        |        |       |
|--|----------------|--------|-------|--------|--------|-------|
|  | Plasma         |        |       | Blood  |        |       |
|  | Beef 1         | Beef 2 | Dog 1 | Beef 1 | Beef 2 | Dog 1 |
|  | mg.            | mg.    | mg.   | mg.    | mg.    | mg.   |
| Quick dry ashing technique<br>as described in text                     | 382            | 355    | 383   | 294    | 302    | 315   |
|  | 381            | 353    | 379   | 294    | 301    | 318   |
|  | 376            | 356    | 379   |        | 301    | 315   |
|  | 376            | 355    | 386   |        | 302    | 318   |
|  |                | 353    | 384   |        | 301    | 313   |
|  |                | 352    | 384   |        |        | 315   |
|  |                | 355    | 387   |        |        | 318   |
|  |                | 355    | 383   |        |        | 315   |
|  |                | 354    | 384   |        |        | 315   |
|  |                | 353    |       |        |        | 318   |
|  | 381            | 354    | 390   |        |        | 319   |
|  | 382            | 354    | 388   |        |        | 316   |
| Van Slyke  | 382            | 353    | 382   |        |        | 316   |
|  | 381            | 354    | 384   |        |        | 316   |
|  |                | 354    |       |        |        | 319   |
|  |                | 354    |       |        |        | 316   |
| Eisenman   | 382            |        | 388   | 291    | 300    | 318   |
|  | 376            |        | 386   | 291    | 303    | 316   |
|  |                |        |       |        | 302    | 319   |
|  |                |        |       |        | 302    | 319   |
|  |                |        |       |        | 303    | 316   |
|  |                |        |       |        | 303    | 318   |
|  |                |        |       |        |        |       |
| Quick dry ashing technique. Recovery of 4.62 mg. of Cl added to sample | 4.62           |        |       | 4.58   |        |       |
|  |                |        |       | 4.56   |        |       |

time, since one sample does not require the full attention of the operator as in the quick digestion technique. After the final titration the beakers should be washed at once to prevent the deposition of oxidized silver.

## SUMMARY

A quick, dry ashing technique for the determination of chlorides in blood and plasma by the titration method of Van Slyke is described. The sample, instead of being wet-digested with nitric acid, is dry-ashed with  $\text{AgNO}_3 \cdot \text{HNO}_3$  and magnesium nitrate in the presence of finely divided asbestos. The time required for ashing is approximately 3 minutes.

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## DEUTERIUM AS AN INDICATOR IN THE STUDY OF INTERMEDIARY METABOLISM

### VIII. HYDROGENATION OF FATTY ACIDS IN THE ANIMAL ORGANISM\*

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We have reported experiments (1) in which unsaturated deuterio fatty acids<sup>1</sup> were isolated from mice fed with saturated deuterio fatty acids. These experiments proved that the animal organism can desaturate fatty acids.

It is of interest, therefore, to determine whether this is a biologically reversible process; that is, whether the organism is also capable of transforming unsaturated into saturated fatty acids. By feeding unsaturated deuterio fatty acids we now have been able to demonstrate their biological conversion into saturated fatty acids.

The chemical preparation of unsaturated deuterio acids necessary for such feeding experiments is one of great difficulty. The material obtained by partial hydrogenation of highly unsaturated fatty acids is not sufficiently homogeneous to justify its use in biological experiments. Linoleic acid, for example, on partial hydrogenation, yielded a mixture. This probably contained, besides oleic acid, other acids with a *trans* (elaidic) configuration or with a double bond at C<sub>11</sub>-C<sub>12</sub>. For our purpose it was preferable to assure the complete absence of such unphysiological acids.

The preparation of unsaturated deuterio fatty acids was satisfactorily effected by a biological process: by the desaturation of saturated deuterio fatty acids by the organism itself. We have

\* This work was carried out with the aid of a grant from the Josiah Macy, Jr., Foundation.

<sup>1</sup> We designate as deuterio compounds those substances which contain more than the normal amount (about 0.02 per cent) of deuterium.

described the isolation of such fatty acids (containing 0.51 and 1.19 atom per cent deuterium) from mice fed with saturated acids containing 8.6 atom per cent deuterium (1).

The quantity (2.74 gm.) of unsaturated fatty acids available from previous experiments was insufficient for our purposes. We therefore repeated the above experiment, feeding a group of eight mice with saturated deuterio acids. An additional 4 gm. of unsaturated deuterio fatty acids was obtained from these animals by the methods previously described. Owing to the sensitivity of the analytical methods for deuterium, the total quantity sufficed for feeding experiments on two mice. The ethyl esters of the acids, together with dry bread, were fed to two mice for a period of 10 days. The animals were then killed and the total fatty acids of each animal were isolated. While the separation of the last traces of saturated acids from the unsaturated fraction is effected only with difficulty (1), the purification of the saturated acids is more easily accomplished by recrystallization from alcohol. The saturated acids thus obtained from both animals contained deuterium in amounts indicative of their origin from the unsaturated fats administered to the animals.

#### EXPERIMENTAL

*Biological Preparation of Unsaturated Deuterio Fatty Acids*—Eight mice were fed for 10 days with bread mixed with 8 per cent of deuterio fatty acids obtained by the complete hydrogenation with deuterium of the fatty acids of linseed oil. These acids contained 11.1 atom per cent deuterium. The isolation of the unsaturated acids from these animals was effected as previously described (1), the last traces of saturated deuterio acids being removed by the repeated addition and precipitation (as lead salts) of natural, saturated fatty acids, 3.967 gm. of unsaturated acids being obtained (iodine number, 115.1; 1.06 atom per cent deuterium). This product, together with 2.74 gm. of unsaturated acids obtained from our previous experiments, was esterified by boiling with ethyl alcohol containing 2 per cent sulfuric acid. The esters were purified by distillation *in vacuo*, 5.67 gm. of a colorless, odorless oil being obtained, containing 0.93 atom per cent deuterium. The residue from the distillation was a brown viscous oil (1.02 gm.) containing 0.40 atom per cent deuterium.

*Feeding of Unsaturated Esters to Mice*—Two mice kept in the same cage consumed, in a period of 10 days, 54.6 gm. of dried ground whole wheat bread and 5.56 gm. of the esters. They received ordinary drinking water. Their weight remained constant during the course of the experiment. After they were killed, a small amount of water was distilled off *in vacuo* from each carcass; this being used to measure the deuterium content of the body fluids.

*Isolation of Total Fatty Acids*—Each carcass was refluxed with 100 cc. of 7 per cent alcoholic KOH for 2 hours and the bones removed from the solution by filtering through glass wool. The bulk of the alcohol was distilled off and the residue was dissolved in water. The alkaline solution was extracted with ether to remove unsaponifiable matter, and the aqueous layer, after acidification with sulfuric acid, was again extracted with ether. The ether was washed with water until neutral and decolorized with a small amount of charcoal. The residue from the ether represented the total fatty acids, 1.3 gm. from Mouse 1 and 1.1 gm. from Mouse 2.

*Fractionation of Fatty Acids*—The acids were dissolved in about 15 times their weight of 95 per cent ethyl alcohol, and an equal amount of lead acetate in the same volume of alcohol was added to the hot solution. The precipitate was filtered off after 24 hours and recrystallized from 95 per cent ethanol. A small amount of material insoluble in hot alcohol was discarded.

The lead salts thus obtained were decomposed by treating them with aqueous HCl and ether. The ether layer, after being washed neutral, was brought to dryness. Yield, 0.452 gm. from Mouse 1 and 0.311 gm. from Mouse 2. Both samples of saturated acids were twice recrystallized from ethyl alcohol (final yield, 0.275 gm. from Mouse 1 and 0.241 gm. from Mouse 2).

The unsaturated acids were isolated from the mother liquors of the lead precipitation and freed from contaminating saturated deuterio acids in the manner described in our previous publication (1).

The four samples of fatty acids thus obtained were dried *in vacuo* before combustion, the water obtained being purified for deuterium analysis (2).

The deuterium content of the water obtained by combustion of the unsaturated acids was determined by refractive index measurement. The deuterium content of the body water and of the water



obtained by combustion of the saturated acids was determined by density measurements, with the submerged float. The apparatus in our laboratory requires 2 cc. of water and is precise to 2 parts in 10 million in the density which corresponds to 0.0002 atom per cent deuterium. In all cases the required volume

TABLE I  
*Deuterium Content of Fatty Acids from Mice*

| Mouse No.<br>(1) | Unsaturated acids<br>(2) | Saturated acids<br>(3) | Deuterium in body<br>fluids<br>(4) |
|------------------|--------------------------|------------------------|------------------------------------|
|                  | <i>atom per cent</i>     | <i>atom per cent</i>   | <i>atom per cent</i>               |
| 1                | 0.25                     | 0.047                  | 0.043                              |
| 2                | 0.10                     | 0.025                  | 0.028                              |

was obtained by dilution of the samples with carefully purified tap water. The determinations were always made in duplicate, the water samples being carried through the whole purification process between determinations. The results are given in Table I. The average deviation of the values in Columns 2, 3, and 4 are  $\pm 0.01$ ,  $\pm 0.001$ , and  $\pm 0.001$  atom per cent respectively.<sup>2</sup>

#### DISCUSSION

The question arises as to whether the deuterium content of the saturated acids can with certainty be ascribed to the saturation of the unsaturated diet fat, or whether other mechanisms exist which might lead to the same result.

<sup>2</sup> It should be noted again that the values given in Table I, as well as all other figures we have given in previous publications, refer to the excess deuterium content relative to normal tap water. To obtain the absolute deuterium content of these samples it would be necessary to add 0.016 per cent to each figure since this is the concentration of deuterium in New York City water (3). The deuterium concentrations in Columns 3 and 4 have been calculated on the assumption that the excess density is due solely to the deuterium present. Dole (4) has recently shown that in commercial tank oxygen, such as we use in our combustion, the concentration of  $O^{18}$  is greater than in the water of Lake Ohio. He found that the water obtained from the combustion of benzene with such oxygen was from 3 to 6 parts per million heavier than his standard lake water. This difference was due to the oxygen isotopes. In this laboratory we have found that water obtained from the combustion of stearic acid has a density of 4 parts per million greater than our standard tap water. It is likely that in this case also the excess density is due to the oxygen isotopes. This factor does not affect the values for the body fluids.

When a deuterio compound is fed to an animal, part of it is oxidized and the heavy water formed is distributed throughout the body fluids (5). All synthetic processes in the animal will now occur in a medium of dilute heavy water, and all compounds formed in the body will contain small amounts of deuterium (6). If, therefore, after the administration of a deuterio Substance A a deuterio Substance B is formed in the animal, two explanations for the deuterium content of Substance B are possible: (a) Substance A was converted into Substance B; (b) a part of the deuterium of the heavy water formed by biological oxidation of Substance A was incorporated into Substance B during its synthesis in the body.

In the latter case the deuterium content of Substance B must be very small, since the  $D_2O$  resulting from the combustion of Substance A will be diluted with the relatively great volume of the tissue fluids. In general, the deuterium content (calculated as atom per cent) of newly synthesized fatty acids will be smaller than the deuterium content of the body fluids. We have already reported data in which it was shown that the deuterium content of the saturated fatty acids is only one-third to one-fourth that of the body fluids when equilibrium has been attained (6).

The saturated acids contained 0.047 and 0.025 atom per cent deuterium, while the deuterium content of the tissue fluids was about the same, 0.043 and 0.028 atom per cent, respectively.

The quantitative data from our experiments make it appear improbable that more than one-third of the deuterium in the saturated acids originated from the body fluids. In the present experiments, in which the deuterium content of the body fluids was originally 0, rising slowly to 0.043 and 0.028 atom per cent after 10 days, the deuterium content of the saturated acids should be even less than one-third that of the body fluids.

We conclude, therefore, that at least two-thirds, and possibly more, of the saturated deuterio acids originated in a conversion (hydrogenation) of the ingested unsaturated acids.<sup>3</sup>

<sup>3</sup> The publication of our experiments on the desaturation of fatty acids (1) occurred before the completion of the study of the synthesis of deuterio fatty acids in the organism (6). It was not possible, therefore, to present the above consideration at that time. In the work on desaturation the high deuterium content of the isolated unsaturated acids (from 2 to 5 times that of the body fluids) excluded any possibility of the deuterium being derived from the tissue fluids.

While the absolute quantity of deuterium in the saturated acids is small, it is large relative to that of the unsaturated acids. The ratio  $D_2$  in saturated acids to  $D_2$  in unsaturated acids is 0.19 in Mouse 1 and 0.25 in Mouse 2. These figures indicate that approximately 20 per cent of the saturated acids have been directly derived from the unsaturated acids.

The data are an additional demonstration of the sensitivity of the method. The saturated fatty acids originally fed to the mice contained 11 atom per cent deuterium. This quantity of deuterium in the course of the two passages through the animal body and its final isolation as fatty acids underwent a dilution of 270 to 440 times. Considering also the dilutions necessary for the analyses, an over-all dilution of 2000- to 3000-fold occurred. Despite this great dilution, the average deviation is not more than 3 per cent.

#### SUMMARY

Deutero unsaturated fatty acids were fed to mice and the saturated fatty acids isolated. The deuterium content of these saturated fatty acids indicated their origin from the ingested unsaturated fatty acids. These data, together with the results of previous work, demonstrate that saturation and desaturation of fatty acids in animals is a physiologically reversible process.

The unsaturated deutero fatty acids fed to our animals were prepared biologically, being isolated from mice fed with saturated deutero fatty acids.

The authors are deeply indebted to Mr. M. Graff for his valuable assistance in the course of these experiments.

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## THE LIPID CONTENT OF RABBIT LEUCOCYTES\*

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The object of the present study was to ascertain what may be considered normal variations in the lipid content of the white blood cells of healthy animals. Following a preliminary communication in 1931 (1) on the phospholipid content of the blood leucocytes of dogs, Boyd in 1933 (2) reported a method for separating leucocytes from blood and gave values for the various lipids in these cells in healthy human adults. It was noted in the latter communication that marked variations occurred in the apparently healthy human adults studied. The calculated standard deviations ranged from 20 per cent to 100 per cent of the respective means, most of the standard deviations being well over 40 per cent of the means. Considerable improvement in technique has been made since that time, both in separating the leucocytes from blood and in the method of analysis. In 1936 Boyd (3) reported on the variations encountered when eight different samples of leucocytes were separated and analyzed from the same sample of human blood and found that the standard deviation of these analyses averaged about 10 per cent of the means for phospholipid and free cholesterol. Although the further lipids were not analyzed at that time, it is probable that a similar percentage standard deviation would have been obtained for total fatty acids and total cholesterol, since these values are estimated directly in the oxidative procedure employed. Other lipid values, such as ester cholesterol, neutral fat, and certain of the fatty acids, would probably have exhibited a greater percentage standard deviation on repeated analyses of different preparations from

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the same blood, because these lipids are calculated from two or more experimentally determined values and hence may present an accumulation of their experimental errors.

Part of the extensive variations in leucocytic lipid values of healthy persons has been shown to be due to variations in the technique of separating and analyzing the white cells. Even after this is taken into consideration, there remains a wide range of values in the results reported for normal persons (2). A further reason for these variations was found (4) to have been the previous calculation of means and standard deviations from a relatively small number of cases. In 1936 (4) the lipid content of white blood cells was recorded for thirty normal human adults, with percentage standard deviations ranging from 29 to 101 per cent of the respective means, but, although these extremes are greater than those found in 1933 (2), most of the percentage standard deviations were lower than those previously found in the smaller series of cases.

In many respects it is difficult properly to standardize experiments on the human subject. The previous occurrence of even minor infections, such as colds, may influence some of the leucocytic lipid values of man, since infection affects the concentration of practically all lipids of the white blood cells and especially that of phospholipid (5). In experiments performed on women, it must be remembered that certain phases of the sexual and reproductive cycles may influence the values of leucocytic lipids (6). Surgical interference, even of a minor character, affects chiefly the phospholipid content but may also alter values for the other lipid fractions (7). Boyd and Wilson (8) showed that in the very young the lipid content of the leucocytes is low, a result which Boyd and Stephens (9) interpreted as being probably due to a relatively greater proportion of lymphocytes in the very young, since they found that the lymphocyte contains less phospholipid and free cholesterol than the polymorphonuclear leucocyte.

The young rabbit was selected for the present experimental study. A colony of thirty such animals was obtained fresh from the farms and housed under standard conditions for a period of 1 month before the leucocytes were analyzed. During this time they were observed at intervals to assure normal behavior and

the absence of infection. The animals ranged in weight from 1400 to 2800 gm., with an average weight of 1750 gm. Differences in weight were not found to have any detectable effect on the lipid values. For example, values for phospholipid averaged 984 mg. per 100 gm. of moist weight in three animals weighing less than 1500 gm., 880 mg. per cent in nine animals weighing 1500 to 2000 gm., 884 mg. per cent in ten animals weighing 2000 to 2500 gm., and 922 mg. per cent in eight animals weighing over 2500 gm. These results at first sight might suggest that the leucocytes of underweight and overweight animals of the same age group (these animals were about 6 months old when obtained) possessed more leucocytic phospholipid than animals of average weight, but the differences will be seen from results to be reported below to be more than accounted for by the normal variations.

To eliminate changes which might occur owing to changes in sexual rhythm, the animals chosen consisted mostly of males, there being twenty-seven of these to three females. It so happened that the average phospholipid of the three female rabbits was higher than that of the twenty-seven males, but the difference was not considered sufficient in so small a series of comparisons as the present to warrant any deductions regarding the effect of sex.

Approximately 50 cc. of blood were found necessary to give sufficient leucocytes to permit a complete, differential lipid analysis. That is to say, such an amount of blood gave enough leucocytes to satisfy the requirements for at least the minimal amounts of lipids capable of being satisfactorily estimated by these methods, such minimal requirements having been stated by Boyd (10). When, for various reasons, less than this amount of blood was obtained and when less than 250 mg. of leucocytes were separated, the analysis was confined to the estimation of phospholipid and free cholesterol, both of which may be determined on the same aliquot of the alcohol-ether extract. Of the first samples of blood withdrawn from these animals, in only nine out of thirty cases was sufficient material obtained to allow a complete analysis. In twenty-one of the thirty animals blood was again taken about 6 weeks later, after hematological studies had revealed a return to normal in the blood picture, and from these twenty-one animals sufficient leucocytes were isolated in ten cases

to give a complete analysis. In all nineteen complete analyses were performed, five of these being duplicates on the same animal, and a total of fourteen animals had at least one complete analysis

TABLE I

*Lipid Composition of White Blood Cells in Normal Young Rabbits*

The results are expressed in mg. per 100 gm. of leucocytes, moist weight.

| Experiment No.     | Total lipid | Composition of total lipid |             |               |                   |              |             |       |      |                |
|--------------------|-------------|----------------------------|-------------|---------------|-------------------|--------------|-------------|-------|------|----------------|
|                    |             | Neu-tral fat               | Fatty acids |               |                   |              | Cholesterol |       |      | Phos-pho-lipid |
|                    |             |                            | Total       | Phospho-lipid | Cholesterol ester | Neu-tral fat | Total       | Ester | Free |                |
| 1                  | 894         | 283                        | 535         | 225           | 41                | 269          | 233         | 61    | 172  | 337            |
| 2                  | 948         | 333                        | 636         | 282           | 38                | 316          | 154         | 56    | 98   | 423            |
| 3                  | 1325        | 830                        | 994         | 162           | 44                | 788          | 207         | 66    | 141  | 244            |
| 4                  | 1361        | 225                        | 786         | 563           | 9                 | 214          | 283         | 13    | 270  | 844            |
| 5                  | 1518        | 478                        | 980         | 460           | 67                | 453          | 283         | 100   | 183  | 690            |
| 6                  | 1599        | 490                        | 980         | 430           | 84                | 466          | 380         | 125   | 255  | 645            |
| 7                  | 1611        | 463                        | 1000        | 510           | 50                | 440          | 321         | 74    | 247  | 777            |
| 8                  | 1685        | 433                        | 1020        | 547           | 62                | 411          | 370         | 92    | 278  | 820            |
| 9                  | 1882        | 482                        | 1110        | 536           | 117               | 457          | 480         | 175   | 305  | 803            |
| 10                 | 2021        | 462                        | 1190        | 714           | 36                | 440          | 453         | 53    | 400  | 1070           |
| 11                 | 2033        | 669                        | 1250        | 527           | 87                | 636          | 487         | 130   | 357  | 790            |
| 12                 | 2116        | 522                        | 1290        | 760           | 33                | 497          | 421         | 49    | 372  | 1140           |
| 13                 | 2155        | 701                        | 1360        | 640           | 54                | 666          | 440         | 80    | 360  | 960            |
| 14                 | 2332        | 660                        | 1570        | 900           | 44                | 626          | 428         | 66    | 362  | 1200           |
| 15                 | 2334        | 533                        | 1390        | 748           | 136               | 506          | 545         | 203   | 342  | 1120           |
| 16                 | 2335        | 684                        | 1460        | 775           | 35                | 650          | 456         | 53    | 403  | 1160           |
| 17                 | 2357        | 767                        | 1460        | 680           | 52                | 728          | 518         | 78    | 440  | 1020           |
| 18                 | 2394        | 558                        | 1452        | 875           | 46                | 531          | 480         | 68    | 412  | 1310           |
| 19                 | 2433        | 907                        | 1600        | 706           | 33                | 861          | 433         | 50    | 383  | 1060           |
| Mean               | 1860        | 552                        | 1161        | 581           | 56                | 524          | 388         | 84    | 304  | 864            |
| Standard deviation | 475         | 177                        | 306         | 202           | 31                | 158          | 111         | 45    | 94   | 295            |
| “ “                | 26          | 32                         | 26          | 35            | 55                | 30           | 29          | 54    | 31   | 34             |
| % of mean          |             |                            |             |               |                   |              |             |       |      |                |

performed upon their blood leucocytes. In the remaining thirty-two extracts only phospholipid and free cholesterol were estimated. Complete analysis by the technique herein employed yields values for the several lipids listed in Table I.

The rectal temperature was taken at intervals for some time before, immediately before, and at periods after the removal of blood for lipid analysis. Although there was a considerable range of body temperature in these apparently healthy animals, it was not possible to draw any definite conclusion regarding a possible influence of this factor. To take for example the first series of thirty analyses performed, the average free cholesterol of eleven animals with rectal temperatures between  $37.8-38.6^{\circ}$  was 323 mg. per cent, while that of nineteen animals with rectal temperatures between  $38.6-39.4^{\circ}$  was 313 mg. per cent.

The hemoglobin concentration of blood was estimated at frequent intervals but no demonstrable relationship could be shown to exist between this and leucocytic lipid values. The red cell count, performed at the same times, similarly did not appear to be connected one way or the other with the concentration of leucocytic lipids. The lipid content of the white blood cells was not influenced by the total leucocyte count, as was similarly found by Boyd and Stephens (9).

Blood was taken in the morning, following a 15 hour fast, by cardiac puncture under aseptic conditions and under light ether anesthesia. Immediately the animal was anesthetized, the heart was punctured and an attempt made to draw off approximately 50 cc. of blood. This was done as quickly as possible, since ether anesthesia affects the lipid content of the leucocytes but not for several hours (11). Blood so obtained was added to flasks containing 1 mg. of heparin (Connaught Laboratories) per 10 cc. of blood, shaken, and centrifuged at full speed for at least 1 hour. By this time in every case the leucocytes had become thoroughly packed and adherent and the edge of the buffy layer had usually separated from the edge of the centrifuge tube and curled up on the surface of the red cells. It was possible in most cases to remove at one time the entire buffy layer by lightly grasping the edge with a pair of cleaned, wide forceps, lifting it, and shaking it gently in the plasma to wash off adherent red blood cells. In other cases the buffy layer was removed in the same manner but bit by bit. The cells were then transferred to a weighed watch-glass and adherent plasma with the few remaining red cells absorbed on strips of cleaned, dry filter paper. The cells were then weighed, ground with cleaned sand, extracted with alcohol-ether, and the



extract analyzed by Bloor's oxidative micromethods as modified by Boyd (12). The use of heparin as an anticoagulant facilitated the separation of the leucocytes in a form readily removed from blood and proved more satisfactory than the anticoagulant salts. This has also been found in further studies on human blood as well as on the blood of rabbits. It is evident from work now in progress that leucocytes from heparinized blood contain fewer lipids per 100 gm. of moist weight than specimens prepared from oxalated samples of the same blood, the leucocytes behaving in this respect like the red blood cells (13).

### *Results*

There were found to be relatively more white blood cells in rabbit blood than in human blood. The average total leucocyte count of the thirty rabbits was 9400 cells per c.mm., the standard deviation of the mean was 1200, the lowest value was 7500, and the highest 11,900. The formula used for calculating the standard deviation in this paper was that previously given (12). The increase in the total leucocyte count was seen to be due to a relative and absolute increase in the lymphocyte content of rabbit blood as against human blood.

The average percentage of neutrophilic leucocytes in the differential count was 48 per cent of the total leucocyte count, the minimal value found being 41, the highest 56, and the standard deviation 4.2 which was 8.8 per cent of the mean. Small lymphocytes averaged 34 per cent of the total count, with a standard deviation of 3.8 which was 11.2 per cent of the mean; the minimal percentage was 27 and the highest 42. Values obtained for large lymphocytes were between 7 and 16 per cent of the total count, with an average of 11 per cent and a standard deviation of 2.9, which was 26.4 per cent of the mean. In about two-thirds of the cases mononuclear leucocytes were found present, the highest being 5 per cent of the total count, the average 2.5 per cent, and the standard deviation 1.2, which was 48 per cent of the mean. The percentage of transitional cells extended from 0 to 6 per cent of the total leucocyte count, with a mean of 3.2 and a standard deviation of 1.8 or 56 per cent of the mean. On the average there were about the same percentages of eosinophiles and basophiles, namely 0.7 and 0.6 per cent respectively of the total count; the

maximal percentage of both types of cells was 2 per cent and the minimal was 0; the standard deviation for eosinophiles was 0.5 and for basophiles 0.4.

The results of the lipid analyses have been given in Table I; only the nineteen complete analyses have been listed. That these analyses probably represented a typical cross-section of the group may be seen from the fact that the mean values for phospholipid and free cholesterol of these nineteen analyses were almost the same as those of the thirty animals in which these two lipids were determined. Thus the mean phospholipid content of the thirty animals was 899 mg. per 100 gm., while that of the nineteen analyses on fourteen animals was 864 mg. per cent. Corresponding values for free cholesterol were 316 and 304 mg. per cent respectively.

Values for total lipid of rabbit leucocytes extended from 894 to 2433 mg. per cent, with a mean of 1860 and a standard deviation of 475. Total lipid was composed on the average of 46 per cent of phospholipid, 30 per cent of neutral fat, 16 per cent of free cholesterol, and 8 per cent of cholesterol esters (calculated as the sum of ester cholesterol plus cholesterol ester fatty acid). In this respect there was seen no striking difference between leucocytes and most of the body tissues.

The total fatty acid content of the white blood cells extended from 636 to 1600 mg. per cent, with a mean of 1161 mg. per cent and a standard deviation of 306. The total fatty acids were composed, on the average, of 50 per cent of phospholipid fatty acids and 5 per cent of cholesterol ester fatty acids, these fatty acid values being calculated from the corresponding values for phospholipid and ester cholesterol by factors previously given (12). The sum of the phospholipid fatty acids plus the cholesterol ester fatty acids subtracted from the total fatty acids gave a value which was termed neutral fat fatty acids. For the time being and in the absence of evidence to the contrary, this fraction may be considered to represent fatty acids from triglycerides, although one must not overlook the fact that it may subsequently be shown to contain fatty acids other than those in glyceride, phospholipid, and cholesterol ester linkages and may even contain some free fatty acid. Neutral fat fatty acids constituted on the average 45 per cent of the total fatty acids. Boyd (6) has

found that when the leucocytes function in removing debris fat from any organ such as the involuting postpartum uterus there occurs an increase in the neutral fat fraction of the white blood cells.

Total cholesterol percentages extended from 154 to 545 mg. per cent, with a mean of 388 mg. per cent and a standard deviation of 111. Total cholesterol was composed on the average of 78 per cent of free cholesterol and 22 per cent of ester cholesterol or combined cholesterol. The ester cholesterol fraction of most body tissues, except those undergoing degeneration (14), is usually not more than 10 per cent of the total cholesterol and in this respect again the blood leucocytes resemble most body tissues.

It will be noted from Table I that the variation encountered in the lipid concentration of rabbit leucocytes was lower than that previously experienced in studies on normal human adults as noted above. The relative variation may be conveniently estimated by determining the percentage standard deviation or the standard deviation expressed as a percentage of the mean. Such values have been listed in the last line of Table I. The most constant lipid values may be seen to be total lipid and total fatty acid, each of which had a standard deviation which was 26 per cent of the respective means. It is particularly interesting that there should be such a relative constancy in total lipid values, since total lipid, by the scheme of analysis herein used, is calculated as the sum of neutral fat plus cholesterol ester fatty acids plus total cholesterol plus phospholipid. One would therefore expect a summation of experimental errors in the values for total lipid in at least some cases, which would tend to give some very high and some very low figures for this lipid. Yet the relative variability of total lipid was less than that of any actual lipid of which it was composed.

There was comparatively little difference in the relative variabilities of all the lipid values except those of ester cholesterol and cholesterol ester fatty acid. The standard deviation of cholesterol ester fatty acid was 55 per cent of the mean and that of ester cholesterol was 54 per cent of the mean. The standard deviation of all the other lipids averaged from 26 to 35 per cent of the respective means, a variation which is comparable to that found among the lipids of blood plasma.

The lipid concentration of the leucocytes of these rabbits can-

not be directly compared with the previous results for human leucocytes (2, 4), since the latter were separated from oxalated or citrated blood which, as previously stated, has been found in studies now in progress to give relatively higher lipid values per 100 gm. of moist leucocytes than those found in heparinized blood as used in the present work. It may be noted, however, that the rabbit leucocytes isolated from heparinized blood contained about 30 per cent more lipids than human leucocytes isolated from oxalated or citrated blood. Obviously, thus, rabbit leucocytes must contain more lipids than human leucocytes. The difference may be seen to be due to rabbit leucocytes containing twice as much or more of neutral fat than human leucocytes and also slightly greater proportions of free cholesterol. The means for ester cholesterol and phospholipid herein found for rabbit leucocytes were about the same as those previously found in human leucocytes but, since heparinized blood was used in the present study, it is probable that rabbit leucocytes contain slightly more of both of these lipids also.

The effect of bleeding on the lipid content of the white cells was not ascertained because of the danger to the life of the animal in taking large amounts of blood at short intervals. The removal of 50 cc. of blood from a rabbit weighing 1750 gm. would be equivalent to taking 2 liters from a man weighing 70 kilos. In the majority of animals the removal of 50 cc. of blood produced an increase in the rectal temperature when the rabbits were examined again 1 week after cardiac puncture, the average increase in temperature being  $0.44^{\circ}$ . At the same time there occurred a fall of 4 to 5 per cent in the concentration of blood hemoglobin and a decrease of about 2 to 3 per cent in the red cell count in the majority of animals, indicating that the bleeding had produced a slight degree of hypochromic anemia. It was anticipated that bleeding would have had a much greater effect on the blood hemoglobin and red cell count than that which was found. The majority of animals showed a slight degree of leucopenia 1 week after bleeding, the average loss in leucocytes being 450 cells per c.mm. The effects of this hemorrhage 1 week after bleeding were thus a slight febrile reaction, a slight degree of hypochromic anemia, and a slight leucopenia with a relative polymorphonuclear leucocytosis, these changes occurring in the majority of animals.

## SUMMARY

The lipid composition of leucocytes from the heparinized blood of thirty normal, young rabbits was estimated by oxidative micro-methods. It was found that rabbit blood contained relatively more leucocytes per c.mm. than human blood, the difference being due to a relative and absolute increase in the percentage of small and large lymphocytes in rabbit blood. A greater constancy was found in the lipids of normal rabbit leucocytes than in those of healthy human subjects. Rabbit leucocytes contained more lipids than human leucocytes, the increase being due chiefly to more neutral fat and free cholesterol.

The following mean values with their standard deviations were found for the concentrations of lipids in the leucocytes of normal rabbits.

|                                    | mg. per 100 gm. |
|------------------------------------|-----------------|
| Total lipid.....                   | 1860 $\pm$ 475  |
| Neutral fat.....                   | 552 $\pm$ 177   |
| Total fatty acids.....             | 1161 $\pm$ 306  |
| Phospholipid fatty acids.....      | 581 $\pm$ 202   |
| Cholesterol ester fatty acids..... | 56 $\pm$ 31     |
| Neutral fat fatty acids.....       | 524 $\pm$ 158   |
| Total cholesterol.....             | 388 $\pm$ 111   |
| Ester       “.....                 | 84 $\pm$ 45     |
| Free        “.....                 | 304 $\pm$ 94    |
| Phospholipid.....                  | 864 $\pm$ 295   |

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# A METHOD FOR DETERMINING THE SOLUBILITY OF GASES IN PURE LIQUIDS OR SOLUTIONS BY THE VAN SLYKE-NEILL MANOMETRIC APPARATUS\*

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The solubility data for gases in liquids from Seidell (1), Landolt-Börnstein (2), or the International Critical Tables (3) are frequently inadequate to furnish the information desired for all temperatures and conditions, particularly where it concerns new or seldom used inorganic and organic gases and liquids, biological fluids, or special solutions which may be used during an investigation.

A rapid and accurate technique for determining solubilities under various conditions of temperature, etc., would be highly desirable to investigators confronted with the need for this information. The Van Slyke-Neill apparatus, which is common to many laboratories, lends itself for such determinations.

The manometric apparatus is based on the principle of extraction of gases from the liquid and measurement of the pressure of the liberated gas. A certain proportion of the gas remains unextracted, however, and is accounted for in Van Slyke and Neill's calculations (4) by the correction factor  $1 + (S\alpha'/(A - S))$ , where  $A - S$  is the volume at which extraction takes place, and  $S$  the volume of the liquid. The  $\alpha'$  in the above formula is equivalent to  $\lambda$ , or the Ostwald solubility expression, and is obtained from the Bunsen absorption coefficient by multiplying by  $(1 + 0.00367t)$ . The Bunsen absorption coefficient,  $\alpha$ , ( $\beta$ , Seidell) is the customary manner in which solubilities are expressed in the International Critical Tables.

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Van Slyke and his collaborators have utilized the manometric apparatus for the determination of the solubility coefficients of carbon dioxide (5), hydrogen (6), nitrogen (7), and oxygen (8) in water and blood. Their calculations involved the use of  $\alpha'$  values for water. Although the values of  $\alpha'$  for water and blood differ slightly, these authors are justified in the use of the water coefficient in their blood determinations, in view of the insignificant effect of slight variations in  $\alpha'$  on the total correction factor. When it is possible, as in the above determinations, to obtain accurate and complete  $\alpha'$  values from the literature, there is little reason to deviate from the technique which these authors have described. Such, however, is not always the case. We were confronted with this lack of information in attempting to utilize the manometric apparatus for the estimation of the solubility of cyclopropane in water, blood, and oils. In the latter case, the solubility of this gas in oil is so great that the unextracted portion is a considerable part of the whole. An appreciable error would be introduced if the  $\alpha'$  values for water were used in this instance, since the oil-water coefficient is 35. There is nothing to prevent the accurate manometric determination of the solubility of such a gas in such a solvent if the distribution between the solvent and the atmosphere over it can be correctly estimated and a correction factor established for the quantity of unextracted gas.

The present study is an attempt to establish a technique and the necessary calculations whereby the quantity of gas remaining in solution in the liquid phase during the extraction process in the Van Slyke-Neill manometric apparatus may be directly determined and incorporated into a general formula which is applicable to the estimation of the solubility of any gas in any solvent.

### *Technique of Solubility Determination*

**Saturation of Fluid**—The liquid may be saturated by any of the ordinary tonometer methods. The simplest procedure is to bubble the pure gas through the liquid with alternate shaking in a water bath at constant temperature. If a tonometer is not available, an ordinary separatory funnel may suffice. By constantly changing the gas over the liquid, other dissolved gases are dispelled. An alternate method is to saturate the liquid at a lower temperature and then equilibrate the system at the temperature of the

bath. By constant agitation, the excess gas dissolved at the lower temperature comes out of solution and raises the pressure in the tonometer. Opening the chamber to the atmosphere momentarily, several times during the shaking in the bath, restores atmospheric pressure.

If the pure gas is not available, it is necessary to analyze for percentage purity. If the quantity of impurity is small and the solubility of the impurity low compared with the gas in question, the error is negligible provided the partial pressure of the main constituent is known; *i.e.*, nitrous oxide, carbon dioxide, or cyclopropane slightly contaminated with oxygen and nitrogen. It is obvious that pure gases give greater accuracy, but for many problems involving the use of commercial gases, a slight impurity in the gas would give results sufficiently accurate for most purposes.

*Introduction of Sample into Extraction Chamber*—The liquid is most accurately transferred to the manometric extraction chamber by the modified Ostwald pipette, with a 3-way cock at the top, described on p. 571 of Van Slyke and Neill's paper (4). This enables one to transfer gas-saturated liquid to the manometric chamber entirely without exposure to air, which would offer chance of escape to the dissolved gas and of absorption to atmospheric gases. Except where extremely accurate data are required, however, the ordinary Ostwald pipette is adequate if handled carefully, particularly for gases with a relatively high solubility coefficient compared with oxygen or nitrogen. It has been found in this laboratory that if the stop-cock bore is large enough so that very little suction is required to draw the liquid into the pipette, solubility figures for carbon dioxide and nitrous oxide obtained with the Ostwald pipette show practically no deviation from those obtained with the modified pipette. This apparently does not hold for a gas with a low solubility such as nitrogen, since we obtained figures which were consistently higher than the accurate value of Van Slyke, Dillon, and Margaria (7) when the ordinary pipette was used for transfer. Apparently, enough oxygen contamination occurs during transfer to effect this error.

After the extraction chamber has been freed of air by evacuation, 1 or 2 cc. of mercury are run up into the cup. The sample should be allowed to flow into the extraction chamber with the



pipette tip surrounded by the mercury. The level of the mercury reservoir should not be lower than necessary to allow the sample to run into the chamber. The pipette and chamber cocks are then closed and the pipette abruptly withdrawn. (If the pipette is withdrawn slowly, mercury will displace a small portion of the sample in the capillary above the chamber cock.) The mercury reservoir is then lowered to the normal low position, and the chamber cock opened slightly to allow mercury to displace the part of the sample remaining in the capillary bore.

*Extraction of Gas from Liquid*—The extraction is done in the usual manner with the mercury level at 50 cc. 3 minutes shaking will generally suffice to establish equilibrium if the liquid is not too viscous.

#### *Calculation of Solubility*

*Pressure of Liberated Gas*—From the manometer reading, after extraction with the mercury level at 50 cc., is subtracted the manometric reading of a "blank" determination made on the same liquid. This gives the pressure of liberated gas in mm. of mercury.

The blank determination is made by extracting some of the liquid (not saturated with the gas) several times and expelling the liberated gas each time. The liquid in excess of the sample size is run out into the cup, the stop-cock bore sealed with mercury, and the mercury level lowered to 50 cc. (The best meniscus is obtained by lowering the mercury and letting it rise to the mark.) After shaking the chamber 2 or 3 minutes to allow the vapor pressure to become equalized, the blank manometer reading is taken. Where a mixture of gases is used and one constituent is absorbed from the total, a suitable blank must be run on the reagent. If organic liquids are used that are water-insoluble, it is best to air-dry the apparatus and introduce dry mercury.

*Derivation of Formula for Calculation of Solubility*—The various terms used below in the derivation of the solubility formula may be defined as follows:

- $v$  = volume of gas extracted per cc. of solution at the partial pressure of the gas (whose solubility is measured) over the liquid in the tonometer, and at the temperature of extraction
- $V$  =  $v$  plus that portion of the gas per cc. which remains in solution after extraction

- $A$  = volume of the extraction chamber (50 cc. in the usual Van Slyke-Neill apparatus)  
 $S$  = size of the sample in cc.  
 $p$  = observed pressure after extraction at the 50 cc. mark minus the blank manometer reading made on the liquid phase  
 $B$  = barometric pressure or the barometric pressure minus the pressure due to slight impurities in the gas  
 $W_s$  = vapor pressure of the liquid at the temperature of saturation; ( $B - W_s$ ) should represent the partial pressure of the gas in question, so that where a mixture of gases is used and the solubility of one of them is to be measured, ( $B - W_s$ ) is replaced by the partial pressure of this gas, which is determined by analysis of the gas mixture used  
 $t_s$  = temperature of saturation  
 $t_e$  = " " extraction  
 $\lambda$  = Ostwald solubility expression—the volume of gas dissolved per unit volume of solvent at a given temperature for any pressure, when the pressure of gas itself minus the vapor tension of the solvent is equal to atmospheric pressure  
 $\alpha$  = Bunsen absorption coefficient—the volume of gas (reduced to 0° and 760 mm.) taken up per unit volume of solvent when the pressure of the gas itself minus the vapor tension of the solvent is 760 mm.

When extraction is complete the observed pressure  $p$  is converted into  $v$  by Equation 1.

$$(1) \quad (B - W_s)v = \frac{p}{S} (A - S) \quad \text{or} \quad v = \frac{p (A - S)}{(B - W_s) S}$$

The volume  $v$  is corrected for the unextracted gas, and thereby converted into  $V$  by Equation 2.

$$(2) \quad V = v + \frac{SVv}{(A - S)} = \frac{(A - S) v}{A - S - Sv}$$

By substituting the value of  $v$  in Equation 1 for  $v$  in Equation 2, the corrected volume  $V$  is obtained in terms of  $p$  in Equation 3.

$$(3) \quad V = \frac{(A - S) p}{(B - W_s) S - Sp}$$

The volume  $V$  is converted to  $\lambda$  by changing the volume at the temperature of extraction to the volume at the temperature of saturation (Equation 4).

$$(4) \quad \frac{(A - S) p}{(B - W_s) S - Sp} \times \frac{273.1 + t_s}{273.1 + t_e} = \lambda$$

|   |  |
|---|--|
| Conversion of observed $p$ to volume of gas at partial pressure of saturation (corrected for unextracted gas) | Factor for correcting volume from temperature of extraction to temperature of saturation |
|---|--|

$\lambda$  is converted into  $\alpha$  in Equation 5.

$$(5) \quad \frac{\lambda}{1 + 0.00367 t_s} = \alpha$$

The solubility of any gas in any liquid or solution may be determined from Equations 4 and 5.

### *Comparative Results*

The solubility data found in the various tables quoted from different investigators are not always in agreement. For comparison, however, the solubilities of a few gases determined by this method are compared in Table I with data taken from the literature. The agreement is fairly good in spite of the fact that only ordinary precautions were taken and that certain refinements in technique, such as those used by Van Slyke and his collaborators in their determinations, were not used in the present experiments.

In a few cases, notably with nitrous oxide, other figures may be found which are not in close agreement with those found by this method. There has been no particular attempt to single out values in the literature which are in agreement with the present data, except in the case of nitrous oxide. With this gas the figures vary over a considerable range, only one of which is given in Table I. It should be emphasized that the present paper is not an attempt to establish new or more accurate solubility constants for the gases which have been studied, but to present a method which is relatively simple that can be used for the determination of solubilities where  $\alpha'$  values are not available. There is every reason to believe, however, that the method could be applied in a more precise manner.

TABLE I

*Comparison of Solubilities Determined by Proposed Method with Those Taken from the Literature*

| Gas            | Liquid | Temperature | Proposed method | Literature  |
|----------------|--------|-------------|-----------------|---|
|                |        | °C.         | $\alpha$        | $\alpha$  |
| Ethylene       | Water  | 25.0        | 0.108           | 0.108 (Winkler (1))<br>0.110 (International Critical<br>Tables (3))                   |
| Nitrous oxide  | "      | 25.0        | 0.549           | 0.545 (Geffcken (1))  |
| " "            | Blood  | 37.5        | 0.416           | 0.417 (Siebeck (9))   |
| Oxygen         | Water  | 25.0        | 0.0281          | 0.0283 (Winkler (1))  |
| Nitrogen       | "      | 25.0        | 0.0149          | 0.0143 ( " (1))<br>0.0150 (Bohr and Bock (1))<br>0.0148 (Van Slyke <i>et al.</i> (7)) |
| Carbon dioxide | "      | 25.0        | 0.753           | 0.759 (Bohr (1))  |

## SUMMARY

A modification of the Van Slyke-Neill method for the quantitative estimation of gases in liquids is presented for determining the solubility of gases in liquids. The principal addition to the former method is that the volume of gas which remains unextracted is corrected for by the technique and method of calculation presented. This makes it possible to determine the solubility of any gas in any liquid or solution without the necessity of resorting to the literature for constants.

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# **A METHOD FOR THE DETERMINATION OF CYCLOPROPANE, ETHYLENE, AND NITROUS OXIDE IN BLOOD WITH THE VAN SLYKE-NEILL MANOMETRIC APPARATUS\***

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The determination of anesthetic gases in blood taken from patients during anesthesia has been reported in several instances (1-3). In each case, however, reservation has been made as to the accuracy of the results because of errors in the vacuum extraction method. The principal source of error has been that the volume of gas determined was not corrected for unextracted gas. This technical difficulty was recognized by the authors cited and the error which was introduced was probably of insufficient magnitude to alter the interpretation of their results significantly. It is felt by the authors of this paper, however, that there is a need for a method by which anesthetic gases can be accurately determined on a single sample without detracting from the accuracy of analysis for carbon dioxide and oxygen.

The determination of blood gases by the Van Slyke-Neill manometric method (4) is based on the principle of extracting the gases from the blood and reagent in a partial vacuum. The gases are separated by absorption with specific reagents, and the volume per cent of the gas in the original blood sample is calculated from the resulting differences in pressures read on the manometer at constant volume.

A correction must be made, however, for the unextracted gas in the liquid. This is accomplished by the factor  $1 + (S\alpha'/(A - S))$  where  $A - S$  is the volume at which extraction takes place, and  $S$  the volume of the liquid. The  $\alpha'$  is equivalent to  $\lambda$ , or the Ost-

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wald solubility expression which may be obtained from the Bunsen absorption coefficient  $\alpha$  by multiplying by  $(1 + 0.00367t)$ .

At the present time it is necessary to make this calculation for each gas from the value of  $\alpha$  for water found in the literature. This procedure is open to two objections. The values found in the literature are not always in close agreement. Secondly, these constants are for pure water, whereas the constants desired are for the acid and alkaline reagents containing blood. For gases with fairly high solubilities, such as the anesthetic gases, there may be considerable variation between the values for pure water and those for aqueous solutions.

By a method recently proposed by one of the authors (5), it is possible to determine accurately the solubility of various gases not only in pure liquids but in solutions as well. The determination is made on the Van Slyke-Neill manometric apparatus, so that the solubility constant for the correction is determined on the same piece of apparatus and under the same condition as is the actual determination of gas in the blood.

Using this method, the authors have determined the solubilities of cyclopropane, ethylene, and nitrous oxide in the various reagents used, and constructed tables of factors for each gas by which the observed pressure is multiplied to give either mm per liter of blood, or volumes per cent in the blood.

#### *General Procedure for Analysis of Blood Containing Anesthetic Gases*

The general procedure for combined carbon dioxide and oxygen in blood (4) is used with a few modifications. After  $p_1$  is read on the manometer and 1 cc. of deaerated alkali run in to absorb carbon dioxide, the solution is again evacuated at the 50 cc. level for 2 or 3 minutes to liberate that part of the anesthetic gas absorbed by the alkali. The level of the liquid is brought to the 2 cc. mark as carefully as before the  $p_1$  reading (40 seconds), so that a standard amount is reabsorbed as Van Slyke and Neill (4) point out with carbon dioxide. The  $p_2$  is then taken and the oxygen absorbent added as usual. The solution is again shaken for 2 to 3 minutes at 50 cc. to liberate the anesthetic gas absorbed by the hydrosulfite reagent. The level is carefully raised to the 2 cc. mark again in 40 seconds and the  $p_3$  reading taken. The anesthetic gas is then determined by difference as in the carbon monoxide determina-

tion (4); *viz.*, the gas is forced out through the cock without loss of liquid and the liquid level brought back to 2 cc. for the  $p_4$  reading. The concentration of gas in the blood is calculated by  $(p_3 - p_4)$  times the factor given in Table III.

To determine whether or not the anesthetic gas is liberated to approximately the same extent after addition of the alkaline reagents, parallel determinations were made—one where the deaerated reagents were saturated with cyclopropane and one with no cyclopropane. The results, in mm. of Hg, are as follows:

| Pressure differences | Deaerated reagents | Deaerated reagents<br>with $C_3H_6$ |
|----------------------|--------------------|-------------------------------------|
| $p_1 - p_2$          | 1.9                | 1.9                                 |
| $p_3 - p_4$          | 1.0                | 0.9                                 |

#### *Error Due to Nitrogen*

If the anesthetic gas diluted with oxygen is administered by the open technique, it is not necessary to correct for nitrogen in the blood if a half hour or more of anesthesia has elapsed. The original level of nitrogen in the blood is 1.2 or 1.3 volumes per cent with an inspired atmosphere of 79 per cent nitrogen. When this high concentration is reduced, the level in the blood must also fall. Although nitrogen continues to enter the blood from the tissues, it is not likely that after half an hour of a nitrogen-deficient atmosphere in the lung that the concentration in the blood will exceed 0.2 or 0.3 of a volume per cent. This being the limit of accuracy of the apparatus with a 1 cc. sample, no correction for nitrogen need be made.

If a closed system with carbon dioxide absorption is used, a careful flushing of the system with oxygen and the anesthetic gas from time to time suffices to reduce the nitrogen content of the blood. If it is desired to take a blood sample shortly after induction, the nitrogen in the blood is probably reduced at least one-half, so that the error would not exceed 0.5 volume per cent.

#### *Influence of Presence of Cyclopropane, Ethylene, and Nitrous Oxide on Accuracy of $CO_2$ and $O_2$ Measurements*

The solubility of the three anesthetic gases was determined for each of the three solutions which are used during the course of the



blood gas determination; *viz.*, Solution 1, 2.5 cc. of acid ferrieyanide reagent plus 1 cc. of blood; Solution 2, the same as Solution 1 plus 1 cc. of 1 N alkali; Solution 3, the same as Solution 2 plus 1 cc. of alkaline hydrosulfite reagent. The solubility was determined by the method referred to above (5). The calculation was made by the formula

$$\frac{(A - S)p}{(B - W_s)S - Sp} \times \frac{273.1 + t_s}{273.1 + t_e} = \lambda = \alpha'$$

|             |  |
|-------------|--|
| where $A$   | = chamber capacity                                       |
| $S$         | = sample size  |
| $(A - S)$   | = volume during extraction                               |
| $p$         | = pressure due to the liberated gas                      |
| $B$         | = barometric pressure                                    |
| $W_s$       | = vapor pressure of solvent at temperature of saturation |
| $(B - W_s)$ | = partial pressure of gas during saturation              |
| $t_s$       | = temperature of saturation                              |
| $t_e$       | = " " extraction   |
| $\lambda$   | = Ostwald solubility expression, equivalent to $\alpha'$ |
| $\alpha'$   | = Van Slyke and Neill expression                         |

The correction factor by which the pressure of liberated gas is corrected for unextracted gas is obtained from the following formula (4).

$$1 + \frac{Sa'}{A - S} = f$$

In Table I are given the values of  $\alpha'$  and  $1 + (Sa'/(A - S))$  for each of the three solutions referred to above. It will be noted that for each gas the solubility ( $\alpha'$ ) decreases as the pH is increased from Solutions 1 to 2 to 3. The decrease in solubility is compensated in each case by the increase in amount of solution, so that the correction factors lie in the same range.

To determine the effect of the slight variation in these factors, the corrections in mm. of mercury may be obtained by assuming a pressure for each gas which is in the range of pressure usually determined: for cyclopropane and ethylene, 25 mm.; for nitrous oxide, 60 mm. When these pressures are divided by the solubility correction factor for each solution, the theoretically observed pressure may be obtained. These figures are given in Table II. The only significant difference is with nitrous oxide between Solutions 1 and 2. This difference of 0.5 mm., however, is equivalent to 0.1

volume per cent, which is within the accuracy of the CO<sub>2</sub> and O<sub>2</sub> determinations.

From this evidence it may be concluded that under these conditions, CO<sub>2</sub> and O<sub>2</sub> may be absorbed in the presence of cyclopropane, ethylene, and nitrous oxide, so that the differences in

TABLE I  
*Constants for Anesthetic Gases in Reagent-Blood Mixtures*

| Solution No. | Reagent-blood mixture  | Cyclopropane |                            | Ethylene  |                            | Nitrous oxide |                            |
|--------------|--|--------------|----------------------------|-----------|----------------------------|---------------|----------------------------|
|              |  | $\alpha'$    | $1 + \frac{S\alpha'}{A-S}$ | $\alpha'$ | $1 + \frac{S\alpha'}{A-S}$ | $\alpha'$     | $1 + \frac{S\alpha'}{A-S}$ |
| 1            | 2.5 cc. acid ferricyanide + 1 cc. blood at 25° ( $A - S = 3.5$ )           | 0.408        | 1.031                      | 0.118     | 1.009                      | 0.643         | 1.048                      |
| 2            | Same as Solution 1 + 1 cc. 1 N alkali at 25° ( $A - S = 4.5$ )             | 0.354        | 1.035                      | 0.110     | 1.011                      | 0.562         | 1.056                      |
| 3            | Same as Solution 2 + 1 cc. alkaline hydro-sulfite at 25° ( $A - S = 5.5$ ) | 0.296        | 1.037                      | 0.093     | 1.011                      | 0.461         | 1.057                      |

TABLE II  
*Observed Pressures of Anesthetic Gases over Reagent-Blood Mixtures*

| Solution No.* | Cyclopropane<br>(25 mm. present) | Ethylene<br>(25 mm. present) | Nitrous oxide<br>(60 mm. present) |
|---------------|----------------------------------|------------------------------|-----------------------------------|
|               | mm. Hg                           | mm. Hg                       | mm. Hg                            |
| 1             | 24.2                             | 24.8                         | 57.3                              |
| 2             | 24.2                             | 24.8                         | 56.8                              |
| 3             | 24.1                             | 24.8                         | 56.8                              |

\* For the composition of the solutions see Table I.

the observed pressures may represent the true pressures of CO<sub>2</sub> and O<sub>2</sub> present within the usual limits of accuracy of the manometric determination.

#### *Calculation of Factors for Cyclopropane, Ethylene, and Nitrous Oxide*

The calculation of factors for the three anesthetic gases by which the observed pressure ( $p_2 - p_1$ ) is multiplied to give concentrations in the blood was made by the formula given by Van Slyke and

Neill (4). The other variable factor given in this formula (beside the solubility correction factor) is the amount of gas reabsorbed when the solution is brought up to the 2 cc. level after extraction, in the arbitrary time of 40 seconds. This factor  $i$  for cyclopropane was found to be 1.01, for ethylene 1.08, and for nitrous oxide 1.03. This indicates that 1, 8, and 3 per cent of the liberated gases, respectively, were reabsorbed when the volume was reduced to 2 cc. It is interesting to note that ethylene has such a high reabsorption

TABLE III  
*Factors for Calculation of  $C_2H_6$ ,  $C_2H_4$ , or  $N_2O$  Content of Blood*

| Temperature | Factors by which mm. of $PC_2H_6$ , $PC_2H_4$ , or $p_{N_2O}$ are multiplied to give |   |   |   |   |   |
|-------------|--|---|---|---|---|---|
|             | mm $C_2H_6$ , $C_2H_4$ , or $N_2O$ per liter of blood                                |   |   | Volume per cent $C_2H_6$ , $C_2H_4$ , or $N_2O$ in blood    |   |   |
|             | Sample 1 cc.<br>$S = 3.5$ "<br>$\alpha = 2$ "<br>$i = 1.01$                          | Sample 1 cc.<br>$S = 3.5$ "<br>$\alpha = 2$ "<br>$i = 1.08$ | Sample 1 cc.<br>$S = 3.5$ "<br>$\alpha = 2$ "<br>$i = 1.03$ | Sample 1 cc.<br>$S = 3.5$ "<br>$\alpha = 2$ "<br>$i = 1.01$ | Sample 1 cc.<br>$S = 3.5$ "<br>$\alpha = 2$ "<br>$i = 1.08$ | Sample 1 cc.<br>$S = 3.5$ "<br>$\alpha = 2$ "<br>$i = 1.03$ |
|             | $^{\circ}C.$   |   |   |   |   |   |
| 20          | 0.1146   | 0.1201  | 0.1191  | 0.2562  | 0.2690  | 0.2673  |
| 21          | 0.1141   | 0.1195  | 0.1186  | 0.2560  | 0.2676  | 0.2661  |
| 22          | 0.1136   | 0.1188  | 0.1181  | 0.2548  | 0.2662  | 0.2649  |
| 23          | 0.1131   | 0.1182  | 0.1176  | 0.2536  | 0.2648  | 0.2637  |
| 24          | 0.1126   | 0.1176  | 0.1171  | 0.2525  | 0.2635  | 0.2626  |
| 25          | 0.1121   | 0.1170  | 0.1166  | 0.2514  | 0.2622  | 0.2615  |
| 26          | 0.1117   | 0.1165  | 0.1162  | 0.2503  | 0.2608  | 0.2604  |
| 27          | 0.1112   | 0.1159  | 0.1157  | 0.2493  | 0.2596  | 0.2594  |
| 28          | 0.1108   | 0.1154  | 0.1153  | 0.2483  | 0.2585  | 0.2584  |
| 29          | 0.1103   | 0.1148  | 0.1148  | 0.2473  | 0.2573  | 0.2574  |
| 30          | 0.1099   | 0.1143  | 0.1144  | 0.2463  | 0.2561  | 0.2564  |

rate compared with carbon dioxide (1.7 per cent), cyclopropane, and nitrous oxide, although its solubility is lower than any of these gases.

With the values of  $1 + (S\alpha/(A - S))$  for  $25^{\circ}$  given in Table I for Solution 3, and similar values determined for other temperatures, and the values of  $i$  given above, the factors in Table III were compiled. The observed partial pressure of any of these three anesthetic gases may be converted to concentration in the blood by the formula,

$$(p_s - p_t) \times f = \text{concentration in blood}$$

where  $f$  is the suitable factor at the temperature of measurement.

## SUMMARY

A method is proposed for determining cyclopropane, ethylene, and nitrous oxide in blood, as well as  $\text{CO}_2$  and  $\text{O}_2$  in the presence of these gases.

Suitable factors for each gas have been compiled and tabulated by which the observed partial pressure may be converted to concentration in the blood.

Corrections have been made for unextracted portions of the gases in the solutions from which they are extracted by direct solubility measurements in these solutions.

It is of interest that the rate of reabsorption in water of ethylene, nitrous oxide, carbon dioxide, and cyclopropane is in the ratio, roughly, of 8, 3, 1.7, and 1, respectively, whereas the ratio of solubilities for the same gases, respectively, is 1, 5, 8, and 2.

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## CHEMICAL AND PHYSICAL STUDIES ON THE ANTI-HEMORRHAGIC VITAMIN

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A description of methods used in concentrating the antihemorrhagic vitamin of the chick was given in preceding papers (1, 2). Since the vitamin had been purified to such extent that 0.5 microgram of the concentrate per gm. of the diet was capable of meeting the requirements of the chick for this factor, it was believed that chemical and physical studies could be made to throw further light on its nature.

The procedure for preventive assay has been already explained (1-3). The methods and results of the present studies are described below. In all cases the concentrate used was the yellow, viscous oil obtained by molecular distillation (2).

### *Methods and Results*

**Nitrogen**—Samples of 24 mg. gave results by the micro-Kjeldahl method (Pregl) which indicated a nitrogen content of 0.23 per cent.<sup>1</sup>

**Sulfur**—Two samples of 9 mg. each were tested for sulfur by the sodium fusion-plumbate method. The tests were negative. Two samples of similar weight were fused in a platinum dish with a mixture of 6 parts of sodium carbonate and 1 part of potassium chlorate. The fused mass was dissolved in distilled water, filtered, acidified with hydrochloric acid, and tested for sulfate with barium chloride. No barium sulfate was precipitated. The concentrate contained no detectable amount of sulfur.

**Phosphorus**—A sample of 9.5 mg. was heated with the carbonate-chlorate mixture in a platinum dish. When all traces of carbon

<sup>1</sup> The author is indebted to Professor D. M. Greenberg for these analyses.

had disappeared, the mass was dissolved in distilled water, filtered, and acidified with nitric acid. Molybdate reagent was then added. No yellow coloration or precipitate formed after heating and prolonged standing. The sample contained no detectable amount of phosphorus. Approximately 0.01 mg. of phosphorus as phosphate was added. A distinct yellow color resulted after heating.

*Carotene*—A sample of 5 mg. was dissolved in chloroform and tested with antimony trichloride reagent. A faint greenish blue color developed, resembling that produced by a known carotene sample. By spectroscopic study of this color an absorption band was detected at  $540\text{ m}\mu$ , the position attributed to  $\alpha$ -carotene (2).

*Xanthophyll*—Partition of a 9 mg. sample between 1 cc. of hexane and 1 cc. of 90 per cent methyl alcohol showed that none of the yellow coloration was transmitted to the alcohol layer. 0.8 mg. of the sample was found in the alcohol layer. Xanthophyll was not present.

*Optical Activity*—A solution of the concentrate in hexane containing 9 mg. per cc. was placed in a 20 cm. polariscope tube. The tube was placed in a Schmidt and Haensch polarimeter and light of wave-length corresponding to the sodium D line passed through it. There was no rotation of the polarized light. The concentrate apparently possessed no optical activity measurable at this concentration.

*Apparent Molecular Weight*—37.6 mg. of the concentrate were dissolved by heating in camphor of sharp melting point at  $176.5^\circ$  (uncorrected). The weight of the camphor in the mixture was found to be 0.2938 gm. The melting point of the mixture was  $166^\circ$  (average of three determinations). The apparent molecular weight of the concentrate was calculated,  $49.8^\circ$  being used as the molal freezing point lowering of camphor, and found to be approximately 600. Since the exact purity of the concentrate is unknown, the determination of molecular weight serves only as a very rough indication of the state of molecular complexity of the concentrate.

*Effect of Phenyl Isocyanate*—This reagent has been used effectively by Olcott (4) to demonstrate the presence of alcoholic hydroxyl groups in the vitamin E molecule. With such groups it forms a urethane derivative. The phenyl isocyanate derivative of vitamin E is quite inactive but becomes active again after alkaline hydrolysis (4).

4.5 mg. of the antihemorrhagic vitamin concentrate were dissolved in 2 cc. of redistilled phenyl isocyanate and heated on a water bath at 100° under a reflux condenser for 1½ hours. The phenyl isocyanate was removed by a current of nitrogen. The residue had the same appearance as the original concentrate. When fed to six hemorrhagic chicks at a level of 1.8 mg. per kilo of diet, it caused the disappearance of all hemorrhagic symptoms within 6 days.

Another portion of concentrate was similarly treated with phenyl isocyanate but the heating was continued for 3 hours. This was tested by preventive assay. A level of 1 mg. per kilo of diet protected the chicks against hemorrhages for a period of 2 weeks, while in the same period of time a group receiving only the basal diet developed a high incidence of hemorrhages. A second preventive assay gave similar results. It is evident that phenyl isocyanate had little effect, if any, on the activity of the antihemorrhagic vitamin. This constitutes a marked difference from vitamin E (4) and indicates that the vitamin either contains no hydroxyl groups or that the urethane derivative can be used by the chicks.

*Effect of 3,5-Dinitrobenzoyl Chloride*—3,5-Dinitrobenzoyl chloride has met with wide usage in the formation of crystalline derivatives of alcohols. A 9 mg. sample of the concentrate was dissolved in 10 cc. of redistilled pyridine and 0.5 gm. of 3,5-dinitrobenzoyl chloride was added. The mixture was heated on a water bath at 100° under a reflux condenser for 2 hours and allowed to stand overnight. Hexane was added and the pyridine was removed by repeated washing with water. When the hexane was evaporated under reduced pressure, the residue appeared the same as the original concentrate, with no evidence of crystals. When fed at levels of 1 and 3 mg. per kilo of diet, the treated concentrate prevented hemorrhagic symptoms. The treatment apparently did not affect the antihemorrhagic vitamin in any way. The results are in agreement with those obtained with phenyl isocyanate.

*Effect of Alcoholic Alkalies*—Since the use of a saponification step would be of great advantage in the purification of the antihemorrhagic vitamin provided conditions could be found under which the vitamin would not be adversely affected, further studies were made of the stability of the vitamin in the presence of alcoholic alkalies.



A 9 mg. sample was dissolved in 25 cc. of methyl alcohol free from aldehydes. 0.2 gm. of potassium carbonate was added. The solution was heated on a water bath at 100° under a reflux condenser for  $\frac{1}{2}$  hour. The concentrate was recovered in hexane and the alcohol washed out with water. At a level of 1 mg. per kilo of diet this treated concentrate failed completely, but at 3 mg. it protected the chicks. Over 50 per cent destruction of the vitamin was evident since a simultaneous test with 0.5 mg. of untreated concentrate diet resulted in no hemorrhagic symptoms.

Two 9 mg. samples were treated as above with the exception that 1 cc. of 10 per cent potassium hydroxide in water was added to each and one sample was kept in an atmosphere of nitrogen during the course of the treatment. Both samples failed at levels of 2 mg. per kilo of diet. In this respect the antihemorrhagic vitamin differs from the antiencephalomalacic chick factor of Goettsch and Pappenheimer (5) which is not destroyed when saponification in purified alcohol is performed in an atmosphere of nitrogen. The yellow color of the samples was not appreciably affected. The great instability of the antihemorrhagic vitamin to saponification in alcohols has been noted previously by Almquist (1) and Dam and Schønheyder (6).

*Effect of 2,4-Dinitrophenylhydrazine*—This reagent for ketonic groups was employed on a 4 mg. sample by adding 1 cc. of glacial acetic acid saturated with the reagent. The solution was heated on a water bath at 100° for 3 hours. It was taken up in hexane and washed repeatedly with water. When free from solvent, no crystals were evident in the residue. The treated concentrate was tested only by the curative procedure. The entire sample added to 1 kilo of diet cured five hemorrhagic birds within 3 days. The activity of the concentrate was not noticeably affected. The vitamin would seem to have no carbonyl group which can combine with 2,4-dinitrophenylhydrazine.

*Effect of Light*—It has already been reported (1) that the vitamin appears stable to visible light (irradiation for 24 hours of a hexane solution by a 500 watt lamp behind a soft glass plate). Further tests were made to study the effect of sunlight. A sample dissolved in hexane was sealed in a glass tube under an atmosphere of carbon dioxide and exposed to direct sunlight. The sample was rapidly bleached and all activity destroyed. A 9 mg. sample

was placed in a glass tube and the solvent removed under reduced pressure. The tube was evacuated to  $10^{-6}$  mm. of mercury for several hours and then sealed off. It was then exposed to direct sunlight for 2 hours. This sample was not bleached but the activity was again completely destroyed. Birds fed this treated concentrate at a level of 2 mg. per kilo of diet developed a high incidence of hemorrhages comparable to results given by birds on the basal diet alone.

Spectrophotometric measurements<sup>2</sup> showed that the concentrate absorbed strongly in the ultraviolet range, but no distinct absorption bands were detected. It seems probable that the vitamin is destroyed by absorption in this range.

*Effect of Bromination*—Brief exposure to small amounts of bromine in the cold diminishes the activity of the vitamin slightly (1). A 4 mg. sample was dissolved in carbon tetrachloride. Bromine in carbon tetrachloride was added until the color of the solution was a distinct red; the solution was allowed to stand for 5 minutes at room temperature and was then washed several times with dilute sodium bicarbonate solution and with water. After the washings the solution was colorless. This preparation was added to the diet at a level of 2 mg. per kilo. Chicks given this diet developed severe symptoms equivalent to those obtained with a group receiving no antihemorrhagic vitamin supplement. The activity of the concentrate was greatly diminished. This result indicates that the vitamin possesses one or more unsaturated linkages.

*Effect of Iodination*—A quantity of concentrate was treated with iodine in a manner similar to the bromination experiment. Last traces of iodine were removed by washing with dilute sodium thiosulfate solution. This preparation was fed to five hemorrhagic chicks at a level of 4 mg. per kilo of diet. All hemorrhagic symptoms disappeared within 3 days. The activity of the concentrate was not destroyed by the iodine treatment, although the yellow color was removed. Iodine apparently does not inactivate the vitamin as readily as does bromine under the same conditions.

*Effect of Perbenzoic Acid*—9 mg. of concentrate were dissolved in 25 cc. of a solution of perbenzoic acid in chloroform and allowed to stand overnight at room temperature. The solvent was re-

<sup>2</sup> The author wishes to acknowledge the assistance of Mr. A. Klose who made these measurements.

moved under reduced pressure and the residue leached with hexane. The hexane solution was washed repeatedly with dilute sodium carbonate solution and with water. The concentrate was completely bleached and its activity destroyed, 2 mg. in a kilo of diet failing to prevent or moderate hemorrhagic symptoms. The effect of perbenzoic acid agrees with the results obtained with bromine in suggesting the presence of unsaturated linkages in the vitamin molecule.

*Color Reactions*—A sample of 9 mg. was dissolved in glacial acetic acid and a drop of concentrated nitric acid added. Upon warming, the solution became colorless. Hexane was added and water-soluble substances removed by washing. The concentrate was then transferred to 90 per cent ethyl alcohol and a drop of potassium hydroxide solution added. A distinct yellow color developed, indicating the presence of a benzene ring structure. A blank test developed no color.

Approximately 10 mg. were taken up in glacial acetic acid. Concentrated hydrochloric acid was added and the mixture heated. A rose color developed, indicating the presence of an indole nucleus. A similar test was obtained with 0.1 mg. samples of tryptophane and of skatole. The test was repeated with sulfuric acid, as in the Adamkiewicz-Hopkins procedure for tryptophane, with similar results.

A few mg. of concentrate were added to 85 per cent phosphoric acid and the mixture warmed. A pink color developed which again indicated the presence of the indole nucleus.

With the "phenol reagent" of Folin and Denis and a solution of the concentrate in alcohol a positive color test was obtained after heating. A blank test remained negative. About 0.1 mg. of tryptophane gave a comparable positive reaction.

#### DISCUSSION

The absence of phosphorus and sulfur, the failure of phenyl isocyanate, 3,5-dinitrobenzoyl chloride, and 2,4 dinitrophenyl hydrazine to affect the activity of the vitamin, the high apparent molecular weight, and the preferential solubility in hydrocarbon solvent suggest that the vitamin is a very complex substance primarily hydrocarbon in structure.

The fact that the color of the concentrate is not affected in the

same manner or degree as its activity indicates that the vitamin is colorless. Particularly significant in this respect are the experiments on the effect of light, the partial bleaching with bromine and activated magnesium oxide (1), the effect of alcoholic alkalies, and the effect of iodination. It has been shown that this coloration may be due to the presence of carotene or a similar pigment which is very difficult to remove by distillation.

The destruction of activity by cold bromination and cold treatment with perbenzoic acid indicates the presence of unsaturated linkages.

Whether or not the color reactions for the indole nucleus are due to the vitamin, of course, cannot be definitely stated at the present time. It is believed, however, that they are sufficiently interesting to be reported, particularly in view of the fact that the vitamin is synthesized during the putrefaction of proteins. The presence of an indole grouping in the concentrate is very definitely indicated by these color reactions. It seems possible that the vitamin may contain such a grouping, although the indole nucleus may comprise only a small portion of the vitamin structure. Further studies will be attempted on the correlation of these color reactions with physiological activity.

#### SUMMARY

Chemical and physical studies of a concentrate of the anti-hemorrhagic vitamin of the chick indicate that it is a complex, colorless, unsaturated substance and markedly unstable to alcoholic alkalies even in the absence of air. Analyses show the presence in the concentrate of a small content of nitrogen and the absence of sulfur or phosphorus. Color tests indicate the presence of the indole nucleus.

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# THE INFLUENCE OF SODIUM GLYCOCHOLATE ON THE ENZYMATIC SYNTHESIS AND HYDROLYSIS OF CHOLESTEROL ESTERS IN BLOOD SERUM\*

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The presence of a cholesterol esterase in blood serum was demonstrated by the finding (1) that a marked esterification of free cholesterol occurs on incubation of untreated serum, but does not occur on incubation of serum which has previously been heated for 1 hour at 55–60°.¹ In studying this reaction further it was of interest to ascertain the effect of bile salts, first, because it is known from many investigations that they influence the enzymatic hydrolysis and synthesis of esters (3, 4) and, second, because it seemed possible that the increased proportion of free cholesterol, usually found in the serum of persons with diseases of the liver, might be associated with the accumulation of bile salts which presumably occurs in such conditions.

In the present investigation the concentration of total and free cholesterol was determined before and after incubation of human and dog serum to which varying amounts of sodium glycocholate had been added. The bile salt exerted a marked effect which was

\* This investigation was made possible by the support of the Josiah Macy, Jr., Foundation.

¹ A similar process appears to take place in tissues. In 1920 Fex (2) described a marked decrease in free cholesterol without significant change in total cholesterol content (i.e., an esterification) on drying liver and kidney at 30–35°. The change did not occur in tissues dried rapidly at 100°. This highly interesting observation was overlooked until recently because it was made incidentally in the course of a long study of methods for the determination of cholesterol.

quite different in the two species. In human serum the normal esterification of free cholesterol was completely inhibited by most concentrations of bile salt studied; there was no significant change in the proportion between the cholesterol fractions in either direction during incubation. As the amount of sodium glycocholate was decreased below a critical level some esterification occurred, but in no case did the reaction go so far as in the control samples to which no bile salt was added. In dog serum, on the other hand, the larger amounts of sodium glycocholate brought about complete hydrolysis of cholesterol esters. As the amount was decreased the splitting became less and less until a level was reached at which the reaction was reversed and some esterification occurred as in the case of human serum.

#### EXPERIMENTAL

A stock solution of sodium glycocholate was prepared by dissolving 1.5 gm. of glycocholic acid (Riedel-de Haën) in NaOH solution sufficient to bring the pH to approximately 7.0. The solution was diluted to 50 cc. (to give 3.0 per cent glycocholic acid) and nine solutions, containing from 1.8 to 0.3 per cent glycocholic acid, were prepared by diluting suitable volumes of the stock solution with distilled water. The solutions were added to serum in the proportion of 1 volume to 4 volumes (0.1 cc. or 0.075 cc. of bile salt solution to 0.4 or 0.3 cc. of serum, respectively, in most experiments) in small test-tubes, which were stoppered tightly and incubated at about 37° for 1 day. Distilled water was substituted for the bile salt solutions in control samples. The concentration of total and free cholesterol was determined in the original serum and in the incubated samples by the method of Schoenheimer and Sperry (5). Six samples of human and nine samples of dog serum were studied. In four of the former and five of the latter ten different concentrations of bile salt were added, as indicated in Chart 1. In the remaining experiments only one to four different concentrations of sodium glycocholate were investigated. Identical experiments were carried out on three samples of human and three of dog serum which had been heated at 55-60° for 1 hour.

All experiments gave essentially the same result for each species. The relevant findings are presented in the form of com-

posite curves in Chart 1 which shows the influence of varying amounts of sodium glycocholate (expressed as glycocholic acid in mg. per cc. of final solution) on the average ratio of combined to free cholesterol.<sup>2</sup> The effect of small amounts of the bile salt was in general the same in both human and dog sera. The normal esterification was inhibited more and more as the

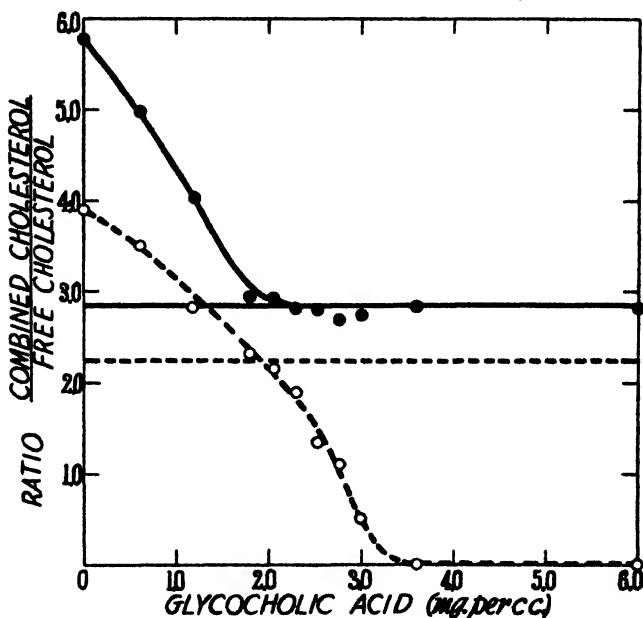


CHART 1. The effect of sodium glycocholate (expressed as concentration of glycocholic acid in the incubated solution) on the enzymatic synthesis and hydrolysis of cholesterol esters. Straight horizontal lines represent the control ratio of combined to free cholesterol. Solid lines represent human serum. Dotted lines represent dog serum. Each point represents the average of four experiments in the case of the human and of five experiments in the case of the dog.

amount was increased until at an average concentration equivalent to about 2 mg. of glycocholic acid per cc. (range 1.7 to 2.3 mg. in both species) the control level was reached; *i.e.*, no esterification of free cholesterol or splitting of cholesterol esters occurred

<sup>2</sup> An increase in ratio over the control level indicates an esterification of free cholesterol; a decrease indicates hydrolysis of cholesterol esters.



during incubation. With larger amounts of sodium glycocholate a marked difference between the two types of serum became apparent. In the human the action of cholesterol esterase was completely inhibited; there was no significant change in the cholesterol proportion in either direction. In the dog, on the other hand, the reaction was reversed: as the amount of the bile salt was increased, a larger and larger proportion of the cholesterol esters was hydrolyzed until at a concentration equivalent to about 2.8 to 3.6 mg. of glycocholic acid per cc. the splitting was complete.

In the experiments with serum which had been heated at 55–60° there was no significant change in the proportion between the cholesterol fractions with any amount of the bile salt in either species. The enzymatic nature of the changes observed in unheated serum was thus demonstrated.

#### DISCUSSION

In 1886 Nencki (6) reported that the addition of bile caused a several-fold increase in the amount of fat split by pancreatic lipase. 5 years later Rachford (7) obtained the same result and showed that bile salt (sodium glycocholate) was responsible for a large part of the effect. These early observations were confirmed and extended in many investigations most of which were concerned with the influence of bile salts on the hydrolysis of fats and univalent esters by various lipases and esterases (references to most of the important publications are given by Oppenheimer (3) and Schmidt (4)). Only three authors studied the influence of bile salts on esterification. Donath (8) found no effect but Hamsik (9) reported that bile and bile salts brought about a marked augmentation of the synthesis of fats by pancreatic lipase. Of particular interest is Nedswedski's recent discovery (10) that emulsified cholesterol is esterified with fatty acids by pancreatic lipase in the presence but not in the absence of bile salts.

Under the conditions employed by the majority of investigators bile salts accelerated the enzymatic hydrolysis of esters to a marked degree. However, as was first emphasized by Loevenhart and Souder (11), bile salts may also inhibit the reaction. The effect may be quite different with closely related esters. Thus under certain conditions the hydrolysis of diacetin is accel-

erated and the hydrolysis of triacetin is inhibited. The situation is further complicated by the finding by several workers that some concentrations of bile salt may accelerate and other concentrations may inhibit the hydrolysis of the same ester by the same esterase. These results may have been associated with changes in pH since Willstätter and his coworkers (12) showed that bile salts accelerate lipolysis only in an alkaline medium; in an acid medium they inhibit.<sup>3</sup> No one has recorded a reversal in the direction of reaction from esterification to hydrolysis, or *vice versa*, simply by the addition of bile salts to a system which otherwise was not altered. Our finding of such an effect in dog serum appears to be unique.

The mechanism by which bile salts accelerate and inhibit enzymatic lipolysis has not been adequately explained though various theories have been proposed. Several of the earlier authors ascribed the phenomenon to the emulsifying action of bile salts. This hypothesis was disproved by the demonstration that the hydrolysis of water-soluble esters and of fats already emulsified in cream or serum, as in the present study, was influenced by bile salts.

Willstätter and his colleagues (12) believed that bile salts and other activators function by forming complex colloidal adsorbates of enzyme and substrate, thereby promoting the reaction. It is difficult to apply this theory to cases, such as the present, in which a reaction is inhibited.

Most investigators have concluded that the effect represents a direct action on the enzyme, the bile salts acting as coenzymes, activators, or inhibitors. This theory may be applied satisfactorily to our result in human serum, since it is only necessary to assume that cholesterol esterase is inhibited by sodium glycocholate. It is difficult, however, to apply it to the finding in dog serum. If the reaction  $\text{cholesterol} + \text{fatty acid}^4 \rightleftharpoons \text{cholesterol}$

<sup>3</sup> It is highly improbable that variations in pH had any influence on the results of the present investigation. The bile salt solutions were adjusted to approximate neutrality and the buffering power of the large excess of serum should have been adequate to prevent appreciable changes in pH. The pH was determined (glass electrode) in samples of serum diluted with water and with the largest amount of bile salt employed in this investigation. A negligible difference was found.

<sup>4</sup> In all probability more than one fatty acid enters into the reaction.

ester + water be considered alone, a direct effect of bile salt on the enzyme catalyzing this reaction, *i.e.* cholesterol esterase, cannot theoretically account for the result, since an enzyme, like any other catalyst, cannot alter the final state of equilibrium in a given reaction,<sup>5</sup> unless it be assumed that the enzyme combines with a sufficient amount of one or more of the reactants to alter significantly the proportion between them. It must be borne in mind, however, that the changes occurring in serum are much more complex than is indicated by this simple reaction. Fatty acids must be furnished by unknown lipids for esterification with cholesterol. Either there is a direct reaction between such lipids and free cholesterol:  $\text{cholesterol} + x\text{-fatty acid} \rightleftharpoons \text{cholesterol ester} + x$ , or the reaction proceeds in two stages: (1) the hydrolysis of *x*-fatty acid and (2) the esterification of cholesterol. In the latter case it is conceivable that the enzyme catalyzing reaction (1) is inhibited by bile salt and that the equilibrium of reaction (2) is thereby shifted in the direction of a splitting of cholesterol esters. It is, however, difficult to account for the difference between human and dog sera on the basis of this hypothesis.

A simple explanation for the findings in dog serum suggested itself. The reversal in direction of reaction could be accounted for by assuming that the bile salt combined with free cholesterol and removed it from the reaction. As more sodium glycocholate was added an equivalent amount of free cholesterol would be removed and the reaction would be shifted in the direction of hydrolysis of cholesterol esters. No pure choleic acids of the conjugated bile acids have been isolated, but it is probable that they exist since the conjugated bile acids share the ability of the free bile acids to keep water-insoluble substances in solution. At the point where complete hydrolysis occurred, the molecular ratio of bile salt to cholesterol was 1.66 to 2.57. However, the foregoing theory does not account for the absence of hydrolysis in human serum in the presence of the larger amounts of bile salt.

It has been impossible to devise any hypothesis which accounts

<sup>5</sup> Terroine (13) claimed that more fat was hydrolyzed at equilibrium in the presence than in the absence of bile salt. He allowed the reaction to continue for nearly a month, but it is not certain that an equilibrium was reached since it was not approached from the opposite direction. No one has studied the kinetics of lipolysis in the presence of bile salt.

satisfactorily for the results in both human and dog sera. It must be remembered that the various lipids concerned exist in the serum in colloidal form, probably in complex combination with other substances, especially proteins. It is open to question, therefore, how far considerations based on the mass law may be applied to a heterogeneous system, in which surface phenomena probably play a dominant rôle. The correct explanation for the findings must await further experimentation.

As noted above the present investigation was suggested in part by the possibility that the decreased ratio of combined to free cholesterol, usually found in the presence of hepatic disease, might be associated with an accumulation of bile salts. As the result of the dog experiments became evident it seemed indeed that a simple explanation for the changes in serum cholesterol in liver disease was at hand. It was only necessary to assume that bile salts exerted the same effect *in vivo* as *in vitro*. The different finding in human serum was, however, not in apparent accord with this hypothesis, although it may be that bile salt accumulation is directly responsible for the decreased proportion of cholesterol esters in hepatic disease by inhibiting the formation of cholesterol esters, which may be going on continuously in the normal organism as it does in normal serum *in vitro*.

#### SUMMARY

Varying amounts of sodium glycocholate were added to human and dog serum and the proportion between the cholesterol fractions was determined before and after incubation. With both species small amounts of bile salt inhibited the normal esterification of free cholesterol somewhat in proportion to the concentration of sodium glycocholate until a point was reached at which no esterification of free cholesterol or hydrolysis of cholesterol esters occurred. With amounts of bile salt above this level a marked difference between human and dog sera was observed. In the former the proportion between the cholesterol fractions remained unchanged with all concentrations studied. In the latter cholesterol esters were hydrolyzed more or less in proportion to the concentration of bile salt until, with the larger amounts, complete splitting occurred.

The enzymatic nature of the reactions was demonstrated by

the finding of no significant change with any concentration of bile salt in either human or dog serum which had been heated for 1 hour at 55–60°.

Various theoretical explanations of the findings are discussed.

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## THE TITRATION CURVES OF OXYGENATED AND REDUCED HEMOGLOBIN

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In 1904 Bohr, Hasselbalch, and Krogh (1) found that changes in the pressure of  $\text{CO}_2$  influenced the oxygen dissociation curves of hemoglobin—as  $p\text{CO}_2$  was increased the affinity of hemoglobin for  $\text{O}_2$  was diminished. In 1914 Christiansen, Douglas, and Haldane (2) discovered that the reciprocal effect also occurs—reduced blood absorbs more  $\text{CO}_2$  than oxygenated blood. Henderson (11) pointed out that this was to be expected in consequence of the equation

$$(1) \quad \frac{(\text{CO}_2) + 7.7}{0.014} = \frac{(\text{Hb})(\text{O}_2)^{1.4}}{(\text{HbO}_2)}$$

which summarized the results. Henderson explained this effect by assuming that the combination with oxygen increases the dissociation constant of a single monovalent acid group in the hemoglobin molecule, so that oxyhemoglobin is a stronger acid than reduced hemoglobin, and binds more base at a given pH.

Hastings, Van Slyke, Neill, Heidelberger, and Harington (10) determined the titration curves of oxygenated and reduced horse hemoglobin by the  $\text{CO}_2$  method; i.e., estimating the pH from the  $\text{BHCO}_3:\text{H}_2\text{CO}_3$  ratio on the basis of the Henderson-Hasselbalch equation, for of course direct measurements of pH with a hydrogen electrode are technically impossible in the case of oxyhemoglobin. Their titration curves extended over the pH range 6.8 to 7.6, and they calculated the change in the dissociation constant of the oxylabile acid group, assuming that it is a single monovalent group which is affected by oxygenation. Hastings, Sendroy, Murray, and Heidelberger (9) found by the same method that carboxyhemoglobin binds as much base as oxyhemoglobin,

and they subsequently conducted a series of direct electrometric titrations on reduced and carboxyhemoglobin, over the pH range 6.2 to 8.6. Their results were of the same order of magnitude as those obtained previously by the  $\text{CO}_2$  method. These experiments confirm Henderson's hypothesis that oxygenation makes hemoglobin a stronger acid.

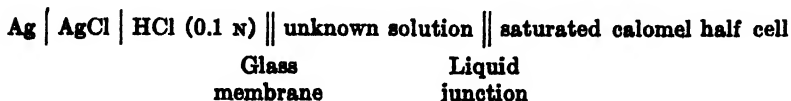
The perfection of the glass electrode has made it possible to obtain titration curves of oxygenated hemoglobin directly, and to reinvestigate the oxygenation-reduction effect. This paper describes a series of titration experiments on oxygenated and reduced hemoglobin, made with the glass electrode over a range of pH from about 4.3 to 9.8 at various ionic strengths.

### *Methods*

*Hemoglobin*—The horse hemoglobin was crystallized by the method of Ferry and Green (7). Concentrated cells from horse blood, kindly furnished by the Massachusetts Antitoxin and Vaccine Laboratory, were diluted with 3 times their volume of cold 1.5 per cent NaCl, and the resulting suspension run through a Sharples centrifuge. The concentrated hemoglobin solution so obtained was crystallized by adding cold 0.1 N HCl until rhomboidal plates appeared close to the isoelectric point, pH 6.6. The crystals were washed twice with cold distilled water. Each washing lasted 6 hours, long enough to remove most of the salt. They were then dissolved by slowly adding the minimum quantity of cold 1.0 N NaOH. The resulting solution was centrifuged to remove stroma, and then recrystallized.

These final crystals were washed twice with distilled water, stirred with cold NaCl solution, centrifuged, and the supernatant solution was used in the experiments. All the operations were carried on in the cold room at a temperature of  $2^\circ$  so as to minimize methemoglobin formation. The hemoglobin was stirred while acid or base was being added to prevent accumulation of local excess, and also to keep it oxygenated. The preparations were always completed 2 or 3 days after the cells were obtained. Comparison of the total nitrogen (Kjeldahl) with the  $\text{O}_2$  capacity (Van Slyke constant volume apparatus) showed that the hemoglobin was only slightly inactivated after being twice crystallized.

*Glass Electrode and Measuring Circuit*—The determinations of pH were based on the E.M.F. of the following combination.



The glass electrode was similar to the one designed by MacInnes (12), except that the glass was thinner. The E.M.F. of the combination was opposed by the E.M.F. drawn from a Leeds and Northrup type K potentiometer, the point of balance being determined by an electron tube bridge similar to the circuit described by Du Bridge (6).

*Manipulations*—A known amount of 0.100 N HCl or of 0.105 N NaOH was added with stirring to a known amount of the cold stock hemoglobin solution. The resulting solution was then divided into two parts and placed in separate tonometers, both of which were evacuated to remove gases. One portion was oxygenated by equilibrating it in the rotating tonometer with cylinder  $\text{O}_2$  previously washed with NaOH solution to remove  $\text{CO}_2$ . The other portion was reduced by repeated evacuation and rotation in an atmosphere of cylinder  $\text{N}_2$  previously bubbled through wash towers containing a solution of anthrahydroquinone sulfonate and  $\text{Na}_2\text{S}_2\text{O}_4$ , made up as described by Fieser (8), to remove  $\text{O}_2$ . Both equilibrations were continued for 10 minutes at room temperature (23–25°) and the measurements were made immediately afterwards. Except in the extreme acid range methemoglobin formation during the course of the measurements was slight or negligible. This was apparent, since, when an oxygenated portion was reduced and then reoxygenated the pH of this reoxygenated hemoglobin solution was the same as that of the original oxygenated solution.

In the extreme acid range where methemoglobin formation was fairly rapid the oxygenated and reduced portions were treated separately in order to avoid standing. They were equilibrated rapidly and the pH measured within 3 to 5 minutes after the addition of acid.

At least three determinations were made upon each portion, and a fresh liquid junction used for each. Over the greater part



of the pH range (4 to 8) the readings were steady and showed a maximum deviation from the mean of 0.15 millivolt (corresponding to 0.0025 pH unit). In the more alkaline range (pH 8.0) there was a drift in E.M.F., sometimes leading to a steady value, sometimes not. In these cases the procedure adopted was to take the average of several readings obtained at the end of 3 minutes after the formation of a fresh liquid junction. For these cases the maximum deviation from the mean was 0.5 millivolt, or 0.008 pH unit.

The electrode was standardized by acetate, phosphate, and borate buffers, made up from tables in Clark (3). The graph of E.M.F. against pH was linear over the range studied. The electrode was checked with the buffers throughout the determinations so as to take account of any changes in the calibration. Results showed a maximum deviation of 0.1 millivolt or 0.0016 pH unit from the mean. All measurements were made at room temperature (23–25°) and the pH of buffers determined by linear interpolation between data given at nearby temperatures.

### Results

The results of five experiments are given in detail in Tables I to V. Experiments 2 and 3 are on the same hemoglobin preparation. The amounts of acid or base added are expressed in terms of milli-equivalents per gm. of hemoglobin. In the case of reduced hemoglobin no correction is made for any inactive protein present, since this is assumed to be methemoglobin, which is known to bind the same amount of base as reduced hemoglobin (9). In the case of oxyhemoglobin, on the other hand, it is necessary to take account of the amount of the inactive material in calculating the base bound. This was done by the following equation

$$(2) \quad B'_o = \frac{B_o - x B_r}{1 - x}$$

where  $B_o$  = total amount of base bound per gm. of Hb (in all forms) in the oxygenated solution;  $B'_o$  = amount of base bound per gm. of active oxygenated Hb;  $B_r$  = amount of base bound by 1 gm. of reduced Hb (assumed to be the same as for the inactive hemoglobin) at the same pH, determined from the data for the

TABLE I  
*Experiment 1*

Total nitrogen = 83.8 mg. per 10 cc.  $\sim$  49.6 gm. of Hb per liter. O<sub>2</sub> capacity = 6.50 volumes per cent  $\sim$  48.4 gm. of Hb per liter. Percentage Hb inactive = 2.3. Salt concentration (stock solution) = 0.154 M NaCl.

| NaCl            | Hb                | Acid (-) or<br>base (+)<br>per gm. Hb<br>total<br>(= reduced)* | pH      |       | Acid (-) or<br>base (+)<br>per gm. HbO <sub>2</sub><br>active* |
|-----------------|-------------------|--|---------|-------|--|
|                 |                   |  | Reduced | Oxy-  |  |
| <i>M per l.</i> | <i>gm. per l.</i> | <i>milli-equivalent</i>  |         |       | <i>milli-equivalent</i>  |
| 0.154           | 49.6              | 0  | 6.675   | 6.590 | 0  |
| 0.151           | 48.6              | +0.042   | 6.935   | 6.84  | +0.042   |
| 0.148           | 47.7              | +0.085   | 7.22(?) | 6.895 | +0.085   |
| 0.140           | 45.0              | +0.214   | 7.915   | 7.760 | +0.215   |
| 0.135           | 43.4              | +0.308   | 8.45(?) | 8.45  | +0.308   |
| 0.137           | 44.1              | +0.260   | 8.17    | 8.08  | +0.260   |
| 0.148           | 48.6              | -0.081   | 5.99    | 6.04  | -0.081   |
| 0.144           | 46.5              | -0.136   | 5.515   | 5.58  | -0.136   |

\* Subtract 0.023 unit to make the zero point coincide with the isoelectric point of reduced Hb, pH 6.81.

TABLE II  
*Experiment 2*

Total nitrogen = 109.0 mg. per 10 cc.  $\sim$  64.5 gm. of Hb per liter. O<sub>2</sub> capacity = 8.58 volumes per cent  $\sim$  64.0 gm. of Hb per liter. Percentage Hb inactive = 1.0. Salt concentration (stock solution) = 0.154 M NaCl.

| NaCl            | Hb                | Acid (-) or<br>base (+)<br>per gm. Hb<br>total<br>(= reduced)* | pH      |       | Acid (-) or<br>base (+)<br>per gm. HbO <sub>2</sub><br>active* |
|-----------------|-------------------|--|---------|-------|--|
|                 |                   |  | Reduced | Oxy-  |  |
| <i>M per l.</i> | <i>gm. per l.</i> | <i>milli-equivalent</i>  |         |       | <i>milli-equivalent</i>  |
| 0.154           | 64.5              | 0  | 6.773   | 6.601 | 0  |
| 0.149           | 62.5              | -0.054   | 6.382   | 6.400 | -0.054   |
| 0.143           | 60.0              | -0.119   | 5.910   | 5.962 | -0.119   |
| 0.148           | 62.0              | +0.070   | 7.20    | 7.046 | +0.070   |
| 0.143           | 59.7              | +0.132   | 7.545   | 7.356 | +0.132   |
| 0.137           | 57.3              | +0.204   | 7.905   | 7.773 | +0.204   |
| 0.132           | 55.3              | +0.270   | 8.360   | 8.246 | +0.270   |
| 0.129           | 53.9              | +0.326   | 8.98    | 9.00  | +0.326   |

\* Subtract 0.006 unit to make the zero point coincide with the isoelectric point of reduced Hb, pH 6.81.

TABLE III  
Experiment 3

Total nitrogen = 68.7 mg. per 10 cc. ~ 40.7 gm. of Hb per liter. O<sub>2</sub> capacity = 5.06 volumes per cent ~ 37.7 gm. of Hb per liter. Percentage Hb inactive = 7.5. Salt concentration (stock solution) = 0.077 M NaCl.

| NaCl            | Hb                | Acid (-) or base (+) per gm. Hb total (= reduced)* | pH      |       | Acid (-) or base (+) per gm. HbO <sub>2</sub> active* |
|-----------------|-------------------|--|---------|-------|---|
|                 |                   |  | Reduced | Oxy-  |   |
| <i>M per l.</i> | <i>gm. per l.</i> | <i>milli-equivalent</i>                            |         |       | <i>milli-equivalent</i>                               |
| 0.077           | 40.7              | 0  | 6.794   | 6.667 | +0.002  |
| 0.074           | 39.2              | +0.106   | 7.448   | 7.250 | +0.109  |
| 0.070           | 37.3              | +0.236   | 8.227   | 8.10  | +0.238  |
| 0.068           | 36.2              | +0.338   | 9.307   | 9.28  | +0.338  |
| 0.074           | 39.4              | -0.087   | 6.100   | 6.110 | -0.087  |
| 0.071           | 37.9              | -0.183   | 5.28    | 5.352 | -0.184  |
| 0.078           | 38.5              | +0.156   | 7.757   | 7.545 | +0.159  |

\* Subtract 0.003 unit to make the zero point coincide with the isoelectric point of reduced Hb, pH 6.81.

TABLE IV  
Experiment 4

Total nitrogen = 113.4 mg. per 10 cc. ~ 67.25 gm. of Hb per liter. O<sub>2</sub> capacity = 9.02 volumes per cent ~ 67.25 gm. of Hb per liter. Percentage Hb inactive = 0.0. Salt concentration (stock solution) = 0.154 M NaCl.

| NaCl            | Hb                | Acid (-) or base (+) per gm. Hb total (= reduced)* | pH      |       | Acid (-) or base (+) per gm. HbO <sub>2</sub> active* |
|-----------------|-------------------|--|---------|-------|---|
|                 |                   |  | Reduced | Oxy-  |   |
| <i>M per l.</i> | <i>gm. per l.</i> | <i>milli-equivalent</i>                            |         |       | <i>milli-equivalent</i>                               |
| 0.154           | 67.25             | 0  | 6.792   | 6.702 | 0   |
| 0.143           | 62.3              | +0.128   | 7.52    | 7.342 | +0.128  |
| 0.139           | 60.6              | +0.172   | 7.710   | 7.555 | +0.172  |
| 0.134           | 58.5              | +0.234   | 8.102   | 7.965 | +0.234  |
| 0.130           | 56.8              | +0.286   | 8.503   | 8.447 | +0.286  |
| 0.129           | 56.0              | +0.312   | 8.815   | 8.790 | +0.312  |
| 0.127           | 55.3              | +0.337   | 9.128   | 9.126 | +0.337  |
| 0.1275          | 55.6              | +0.321   | 8.897   | 8.898 | +0.321  |
| 0.148           | 64.6              | +0.063   | 7.054   | 6.988 | +0.063  |
| 0.148           | 64.7              | -0.059   | 6.378   | 6.348 | -0.069  |
| 0.146           | 63.6              | -0.088   | 6.136   | 6.157 | -0.088  |
| 0.144           | 62.9              | -0.104   | 6.003   | 6.034 | -0.104  |
| 0.138           | 60.5              | -0.167   | 5.490   | 5.576 | -0.167  |
| 0.132           | 57.7              | +0.262   | 8.323   | 8.182 | +0.262  |
| 0.125           | 54.4              | +0.370   | 9.510   | 9.510 | +0.370  |

\* Subtract 0.003 unit to make the zero point coincide with the isoelectric point of reduced Hb, pH 6.81.

reduced Hb in the same experiment; and  $x$  = fraction of total Hb in the inactive form.

TABLE V  
*Experiment 5*

Total nitrogen = 131.44 mg. per 10 cc.  $\sim$  77.95 gm. of Hb per liter. O<sub>2</sub> capacity = 9.90 volumes per cent  $\sim$  73.8 gm. of Hb per liter. Percentage Hb inactive = 5.3. Salt concentration (stock solution) = 0.5 M NaCl. Hb concentration throughout titration = 52.0 gm. per liter. NaCl concentration throughout titration = 0.333 M.

| Acid (-) or base (+)<br>per gm. Hb<br>total (= reduced)* | pH      |       | Acid (-) or base (+)<br>per gm. HbO <sub>2</sub> active* |
|--|---------|-------|--|
|  | Reduced | Oxy-  |  |
| <i>milli-equivalent</i>                                  |         |       | <i>milli-equivalent</i>                                  |
| -0.130   | 6.072   | 6.055 | -0.130   |
| -0.258   | 5.16    | 5.188 | -0.259   |
| -0.390   | 4.610   | 4.618 | -0.392   |
| -0.452   | 4.415   | 4.41  | -0.453   |
| -0.514   | 4.28    | 4.28  | -0.514   |
| -0.419   | 4.525   | 4.525 | -0.420   |
| -0.323   | 4.842   | 4.860 | -0.324   |
| +0.171   | 7.86    | 7.725 | +0.172   |
| +0.331   | 9.350   | 9.355 | +0.331   |
| +0.254   | 8.545   | 8.45  | +0.254   |
| +0.288   | 8.910   | 8.890 | +0.288   |
| +0.292   |         | 8.99  | +0.292   |
| +0.311   | 9.130   | 9.130 | +0.311   |
| +0.357   | 9.465   | 9.480 | +0.357   |
| +0.407   | 9.80    | 9.80  | +0.407   |
| +0.350   | 9.410   | 9.410 | +0.350   |
| -0.172   | 5.69    | 5.80  | -0.173   |
| -0.224   | 5.32    | 5.430 | -0.225   |
| -0.064   | 6.541   | 6.430 | -0.063   |
| +0.070   | 7.295   | 7.130 | +0.072   |
| 0  | 6.910   | 6.795 | +0.001   |
| +0.208   | 8.14    | 8.043 | +0.209   |
| +0.131   | 7.66    | 7.51  | +0.133   |

\* Add 0.018 unit to make the zero point coincide with the isoelectric point of reduced Hb, pH 6.81.

The total hemoglobin present in all forms was determined by the Kjeldahl method, a factor of 5.93 (15) being used to convert gm. of N to gm. of Hb. The amount of active hemoglobin

was based on the  $O_2$  capacity determined by a Van Slyke constant volume apparatus, with 66,800 as the molecular weight of hemoglobin, and assuming that 4 molecules of  $O_2$  combine with 1 molecule of Hb.

Since the original stock solutions did not contain exactly isoelectric hemoglobin, the zero values for milli-equivalents of base bound per gm. of Hb in Tables I to V do not correspond to the isoelectric condition. At the foot of each table is given the amount of base to be added or subtracted from the amount tabulated to make the zero value correspond with the isoelectric point of reduced hemoglobin, pH 6.81 (10).

In most of the experiments the concentration of Hb and NaCl varied somewhat over the titration curve, owing to the addition of variable volumes of standard acid or base for different pH values. In the last experiment (Fig. 1, Table V) this was obviated by the addition of a suitable amount of  $H_2O$  together with the acid or base in order to make the total final volume the same in every case.

#### DISCUSSION

*Nature of Groups Involved*—The results show that between pH  $\cong$  6.1 and pH  $\cong$  9.0 oxyhemoglobin is a stronger acid than reduced and binds more base at a given pH, whereas between pH  $\cong$  4.5 and pH  $\cong$  6.1 oxyhemoglobin is a weaker acid. Above pH about 9 and below pH about 4.5, on the other hand, there is no observable difference between the two forms. In what follows we shall refer to the portion of the titration curve between pH 6.1 and 9.0 as the "alkaline loop," and the portion between pH 4.5 and 6.1 as the "acid loop." We may say, therefore, that in the alkaline loop the effect of oxygenation is to increase the acid dissociation constants of one or more oxylabile groups, while in the acid loop, on the other hand, its effect is to diminish them. This implies that at reactions between 4.5 and 6.1 the familiar Bohr effect of the alkaline range (according to which oxygen affinity increases with pH) should be reversed. Actually Ferry and Green (7) found that this effect is reversed below a pH of about 6.5.

It would appear that the groups dissociating in the range of the alkaline loop are free amino or imino groups (see Cohn (4),

Table III). In any case, these groups must be relatively near the hematin nucleus in order to be affected by oxygenation. The same is true of the other groups, whatever they may be, which dissociate in the acid loop and are oppositely affected by oxygena-

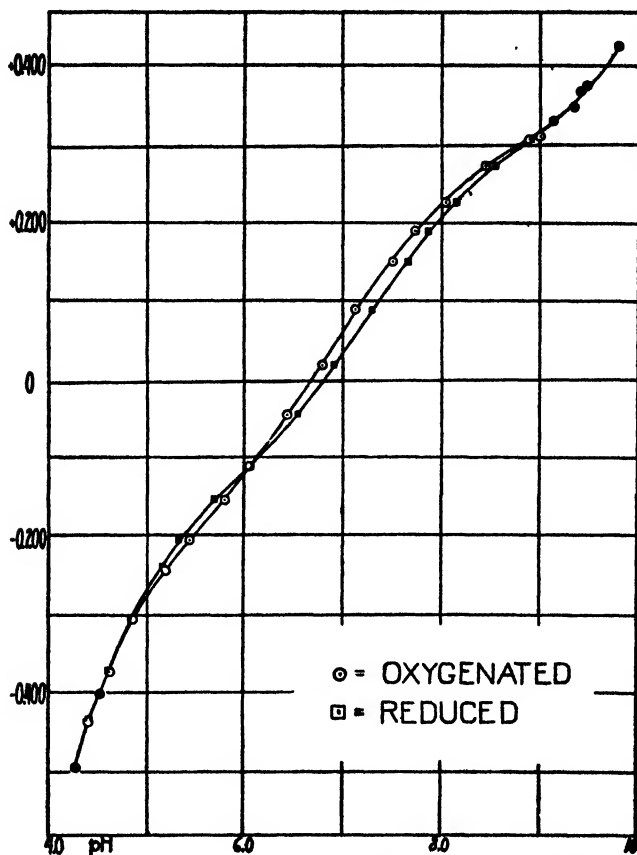


FIG. 1. Graph of Experiment 5. Milli-equivalents of base per gm. of hemoglobin (ordinates) are plotted against pH (abscissa).

tion. It would also appear that these may be either imidazole groups of histidine, or the second carboxyl groups of dicarboxylic acids.<sup>1</sup> Since these two kinds of groups have different tempera-

<sup>1</sup> A recent analysis of the titration curve of hemoglobin has been given by Cohn and Green (5).

ture coefficients, it would be of interest in this connection to know the effect of temperature on the phenomenon. Certainly it is reasonable to expect that the groups affected in the alkaline loop are of a different nature from those in the acid loop, since oxygenation affects them oppositely and because of the range of pH.

*Number of Groups Involved and Magnitude of Shift in  $pK'$* —Hastings and his collaborators (10) have attempted to account for their data, obtained by the  $\text{CO}_2$  method, by assuming that in the alkaline loop only one acid group is affected by oxygenation, and they have calculated the necessary shift in  $pK'$  to account for the

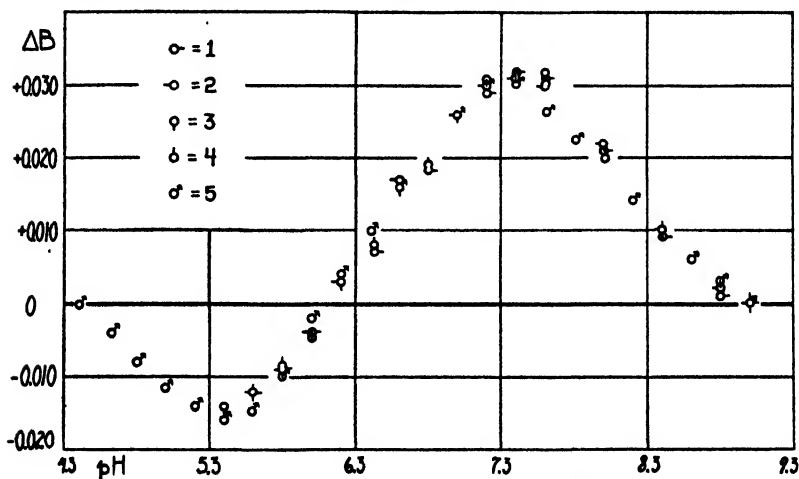


FIG. 2. Difference in base bound (milli-equivalents per gm.) between oxygenated and reduced hemoglobin as a function of pH for Experiments 1 to 5 given in Tables I to V.

effect. In Fig. 2 are shown the differences in base bound by the reduced and oxygenated hemoglobin as a function of pH. The maximum value in the alkaline loop is 2.2 equivalents per mole, assuming a molecular weight of 66,800, and this would indicate that at least three amino groups are affected. The actual number is probably larger than this. Hastings' calculations were based on the old value for the molecular weight of Hb—16,800. His data, recalculated on the basis of a molecular weight of 66,800, also indicate that at least three groups must be affected. A similar consideration applied to the acid loop tells us nothing, since

the maximum difference is 1 equivalent per mole of Hb, and we know that in any case at least one group must be involved. In view of the complications of the situation—the overlapping of effects of different groups, the number of groups possibly affected, and  $pK'$  shifts of different magnitudes—it seems futile to attempt any further analysis of this sort.

*Effect of Ionic Strength*—The data already presented show that over the range studied ionic strength as determined either

TABLE VI  
*Analysis of Experimental Data on Titration of Oxygenated and Reduced Hemoglobin*

| Experiment No.* | Hb         | NaCl       | Maximum $\Delta B$ per gm. Hb | pH of maximum $\Delta B$ | pH of meeting of curves, alkaline side | pH of crossing | pH of meeting of curves, acid side |
|-----------------|------------|------------|-------------------------------|--------------------------|--|----------------|------------------------------------|
|                 | gm. per l. | $M$ per l. | milli-equivalent              |                          |  |                |                                    |
| 1               | 49.6       | 0.154      | 0.032                         | 7.45                     |  | 6.12           |                                    |
| 2               | 64.5       | 0.154      | 0.031                         | 7.43                     | 8.9                                    | 6.12           |                                    |
| 3               | 40.7       | 0.077      | 0.032                         | 7.50                     | 8.9                                    | 6.10           |                                    |
| 4               | 67.2       | 0.154      | 0.032                         | 7.45                     | 8.9                                    | 6.15           |                                    |
| 5               | 52.0       | 0.50       | { 0.031<br>-0.016†            | 7.30                     | 9.0                                    | 6.08           | 4.5                                |
|                 |            |            |                               | 5.50                     |  |                |                                    |
| 6               | 31.1       | None added |                               |                          |  | 6.10           |                                    |
| 7               | 43.7       | 0.5        |                               |                          |  | 6.13           |                                    |
| 8               | 41.5       | 1.0        |                               |                          |  | 6.12           |                                    |
| 9               | 50.0       | 0.5        |                               |                          | 8.9                                    |                |                                    |
| 10              | 50.0       | 1.0        |                               |                          | 9.1                                    |                |                                    |

\* Experiments 2 and 3; 6, 7, and 8; and 9 and 10, were on the same hemoglobin preparations.

† The negative value belongs to the "acid loop."

by Hb or NaCl has little if any effect on the points of crossing or meeting of the curves, or on the maximum value of difference in base bound between the oxygenated and reduced Hb (see Fig. 2). There is some indication that the pH corresponding to the maximum difference in base bound may vary slightly with the salt concentration, and this variation agrees both in magnitude and in direction with that found by Hastings and his collaborators (10). The absence of any effect of ionic strength on the points of crossing or meeting of the curves is confirmed by additional



observations (Experiments 6 to 10) over a narrower pH range, but extending up to greater values of ionic strength. An analysis of the entire situation is presented in Table VI.

It is probably to be expected that the points of crossing and of meeting of the curves should be independent of ionic strength, since wherever the amounts of base bound are the same, the numbers of charges are likewise the same for the oxygenated and reduced hemoglobin molecules, and the effect of salts on their activities should therefore be very nearly the same also.

*Theoretical Interpretation*—As we have seen, it was pointed out by Henderson on the basis of Equation 1 that the effect of oxygenation in increasing the acid dissociation constant of hemoglobin in the alkaline loop implies a reciprocal effect involving a decrease of oxygen affinity with hydrogen ion concentration in the same region. In the acid loop, where the effect of oxygenation is reversed, we should expect the effect of hydrogen ion concentration on the oxygen affinity to be reversed also. At the pH of the point of crossing, therefore, the oxygen affinity should be a minimum.

This result may be derived in more exact form on the basis of a general treatment. We may regard all the dissociable groups in the hemoglobin molecules as acid groups and express their dissociation in terms of acid dissociation constants. Further, we may separate the molecules into five main divisions designated 0, 1, 2, 3, 4, according to the number of oxygen molecules with which they are combined. We will consider first division 0, comprising the completely reduced hemoglobin molecules. These will be represented by a variety of ionic forms whose relative proportions are determined at any given pH. We may class these ions according to the number of hydrogen ions dissociated, or equivalents of base bound, by each: for members of the first class the number is 0; for members of the second class, 1; for members of the  $n$ th class,  $n - 1$ . In general in any class there will be a number of different ionic species characterized by the identity of the particular groups ionized. Members of any particular species of the  $n$ th class will be in equilibrium with the members of a corresponding species of the  $(n - 1)$  class in accordance with a relation

$$(3) \quad r_n = \frac{kr_{n-1}}{H}$$

where  $r_n$  and  $r_{n-1}$  denote the concentrations of the ions of the two species,  $H$  refers to hydrogen ion activity, and  $k$  is a constant. It is convenient to express the concentration of the ions of every species in terms of the concentration of the ions of an arbitrarily chosen species of the first class,  $r_1^*$

$$(4) \quad r_n = \frac{k_{rn} r_1^*}{H^{n-1}}$$

The concentration of all ions of the  $n$ th class is then

$$(5) \quad R_n = \sum r_n = \frac{r_1^*}{H^{n-1}} \sum k_{rn} = \frac{r_1^*}{H^{n-1}} K_{rn}$$

where the summation extends over all species of this class and  $K_{rn} = \sum k_{rn}$ ; and the total concentration of ions of all species and all classes is

$$(6) \quad R = r_1^* \sum_n \frac{K_{rn}}{H^{n-1}}$$

Since the concentration of base bound by ions of the  $n$ th class is  $(n-1)R_n$ , it follows that the total number of equivalents of base bound per mole of reduced hemoglobin is

$$(7) \quad B_r = \frac{\sum_n \frac{(n-1) K_{rn}}{H^{n-1}}}{\sum_n \frac{K_{rn}}{H^{n-1}}}$$

This may be alternatively written as

$$(8) \quad B_r = \frac{d}{d \text{pH}} \log_{10} \sum_n \frac{K_{rn}}{H^{n-1}}$$

Exactly the same treatment is applicable to each of the other four divisions, and we may use the same expression for the base bound, if we replace  $r$  ( $R$ ) by  ${}_1x({}_1X)$ ,  ${}_2x({}_2X)$ ,  ${}_3x({}_3X)$ , or  ${}_4x({}_4X)$

and  $B_r$  by  $B_1, B_2, B_3$ , or  $B_4$  according as we refer to divisions 1, 2, 3, or 4, respectively.<sup>3</sup>

The oxygen equilibrium may be formulated in terms of  $r_1^*$ ,  ${}_1x_1^*$ ,  ${}_2x_1^*$ ,  ${}_3x_1^*$ ,  ${}_4x_1^*$ , and  $p$ , the partial pressure of oxygen,

$$(9) \quad \frac{{}_1x_1^*}{r_1^*} = l_1 p, \quad \frac{{}_2x_1^*}{r_1^*} = l_2^2 p^2, \quad \frac{{}_3x_1^*}{r_1^*} = l_3^3 p^3, \quad \frac{{}_4x_1^*}{r_1^*} = l_4^4 p^4$$

where the four  $l$ 's are constants, independent of pH, since they describe the equilibria of individual ionic species.<sup>3</sup> Hence, on the basis of Equation 6,

$$(10) \quad \frac{{}_1X}{R} = L_1 p, \dots \frac{{}_4X}{R} = L_4^4 p^4$$

where

$$L_1 = \frac{\sum_n \frac{K_{1n}}{H^{n-1}}}{\sum_n \frac{K_{rn}}{H^{n-1}}} l_1, \dots L_4^4 = \frac{\sum_n \frac{K_{4n}}{H^{n-1}}}{\sum_n \frac{K_{rn}}{H^{n-1}}} l_4^4$$

From this we obtain by differentiation and the use of Equation 8 and the corresponding equations for the oxygenated forms

$$(11) \quad \frac{d}{dpH} \log_{10} L_1 = B_1 - B_r, \dots \frac{d}{dpH} \log_{10} L_4^4 = B_4 - B_r$$

The quantity  $B_4 - B_r$  is the observed difference in base bound per mole between completely oxygenated and completely reduced

<sup>3</sup> Of course we must remember that in the case of incompletely oxygenated hemoglobin, the ions of any species will be further divisible into a number of different types, or subspecies, distinguished by the particular oxygen-combining groups oxygenated. The number of such subspecies is uniformly four for division 1, six for division 2, and four for division 3. It is easy to show, however, that the proportion of ions of a given species belonging to each subspecies is constant, and from this it follows that the equations apply as given for all four divisions and that we may disregard distinctions between the partially oxygenated ions due to the particular groups oxygenated.

<sup>3</sup> In accordance with foot-note 2,  ${}_1x_1^*$ ,  ${}_2x_1^*$ , and  ${}_3x_1^*$  will be the sum of four, six, and four subspecies concentrations respectively, each distinguished by the identity of the oxygen-combining groups oxygenated. Each of these subspecies will have its own oxygen dissociation constant, and the  $l$ 's given above are derived from these.

hemoglobin at any given pH. In order to relate this to the oxygen affinity it is necessary to consider the expression for the percentage saturation  $y$ . In its most general form this may be written

$$(12) \quad y = \frac{L_1 p + 2L_2 p^2 + 3L_3 p^3 + 4L_4 p^4}{4(1 + L_1 p + L_2 p^2 + L_3 p^3 + L_4 p^4)}$$

Ferry and Green (7) have shown that the curves obtained by plotting  $y$  against  $p$  at different values of pH may all be made to coincide by changes of scale of  $p$ . This implies that the relative values of  $L_1$ ,  $L_2$ ,  $L_3$ , and  $L_4$  remain unaltered with changes of pH. Consequently the percentage saturation  $y$  is determined completely by the product  $L_4 p$ , or, in other words, the oxygen pressure required to produce a given percentage saturation is inversely proportional to  $L_4$ .  $L_4$  therefore may be taken as defining the oxygen affinity, which we may denote by  $A$ , and it follows from Equation 11 that

$$(13) \quad \frac{d}{dpH} \log_{10} A = \frac{\Delta B}{4}$$

where  $\Delta B$  is  $B_4 - B_r$ , the difference of base bound between completely oxygenated and reduced hemoglobin. Wherever, therefore, the titration curve for oxygenated hemoglobin lies above that for reduced hemoglobin (as in the alkaline loop), the oxygen affinity increases with pH; wherever the reverse is true (as in the acid loop), it decreases; wherever the curves cross, it is a maximum or minimum; wherever they coincide, it is constant.

Equation 11 may be integrated to obtain relative values of the oxygen affinity if  $\Delta B$  is known as a function of pH. This has been done graphically by means of a planimeter on the basis of the data of Experiment 5. Relative values of the oxygen affinity so obtained are represented by the curve in Fig. 3.<sup>4</sup> Observed values of the relative oxygen affinity, taken from the data of Ferry and Green (7), are shown by the circles, and are in fairly good agreement with the curve. The relatively small discrepancy must be attributed either to experimental errors, inaccuracy of

<sup>4</sup> It is evident from Fig. 2 that the use of any of the other experiments would yield almost exactly the same result.

Ferry and Green's generalization as to the superposability of the oxygen dissociation curves, or to changes in the activity coefficients of the individual ions with pH. It should be pointed out that it is difficult to obtain complete reduction of the hemoglobin at strongly alkaline reactions, owing to the high oxygen affinity, and it is possible, therefore, that the observed values of  $\Delta B$  may be too small in the strongly alkaline range.

It is of interest to note that it follows from Equation 11 and Ferry and Green's observation on the superposability of the curves that  $B_1 - B_7 = B_2 - B_1 = B_3 - B_2 = B_4 - B_3$ . This means

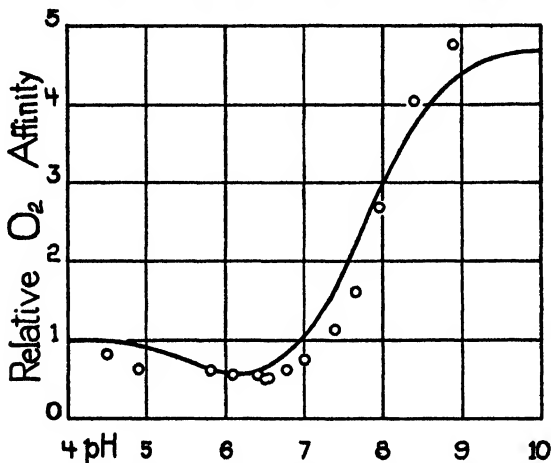


FIG. 3. Relative values of the oxygen affinity of hemoglobin as a function of pH. The smooth curve is calculated by Equation 11 from the data of Table V; the circles are direct experimental values taken from Ferry and Green (7).

that the shift in base bound due to each successive step in the oxygenation is the same. This deduction is in agreement with experimental results of Van Slyke *et al.* (14). It suggests that each heme is associated with the same set of free acid and basic groups, and that the hemoglobin molecule may be thought of as built up of four closely similar parts.

Pauling (13) has recently given a very satisfactory interpretation of the oxygen dissociation curves of hemoglobin based on the assumption that the four hemes are arranged at the corners of a square and that there is an effect on the oxygen dissociation con-

stant of each due to the oxygenation of an adjacent heme but not to that of the one situated at the opposite corner. This leads to the equation

$$(14) \quad \frac{v}{100} = \frac{Lp + (2\alpha + 1)L^2p^2 + 3\alpha^2L^3p^3 + \alpha^4L^4p^4}{1 + 4Lp + (4\alpha + 2)L^2p^2 + 4\alpha^2L^3p^3 + \alpha^4L^4p^4}$$

where  $L$  denotes the oxygen dissociation constant of a single heme when the adjacent hemes are unoxygenated and  $\alpha$  (independent of pH) measures the effect on  $L$  of the oxygenation of one adjacent heme. This equation fits the facts very exactly. It is of course a special case of Equation 12 and is in accordance with Ferry and Green's observation on the superposability of the curves at different pH values.  $L$  corresponds to  $L_1/4$  in Equation 11, and its change with pH is therefore given by

$$\frac{d}{dpH} \log_{10} L = \frac{\Delta B}{4}$$

If we denote by  $\Delta F$  the free energy increase accompanying the oxygenation of a single heme, then

$$\frac{d\Delta F}{dpH} = -2.3026 \frac{\Delta B \times RT}{4}$$

By graphical integration on the basis of the data of Experiment 5 we find that  $\Delta F$  decreases (at 25°) by 1190 calories between pH 6.05, the point of crossing of the curves, and pH 9.13, the point at which the curves come together on the alkaline side; and by 330 calories between pH 6.05 and 4.58, the point at which the curves come together on the acid side.

#### SUMMARY

By the use of the glass electrode titration curves of oxygenated and reduced hemoglobin have been obtained over a range of pH from about 4.3 to 9.5. Between pH 6.08 to 6.15 and pH 4.5 reduced hemoglobin is a stronger acid than oxyhemoglobin; between pH 6.08 to 6.15 and pH 8.9 to 9.1 the opposite is true. The difference in base bound between reduced and oxyhemoglobin at a given pH is little if at all affected by ionic strength in the range studied. An expression has been developed by which it is possible

to predict with fair success the relative values of the oxygen affinity as a function of pH from the data giving the two titration curves. The derivation of this expression presupposes only the most general feature of the oxygen dissociation curves; namely, that the curves obtained at different values of pH may all be made to coincide by a change of scale of the partial pressure of oxygen. If we accept Pauling's interpretation of the oxygen dissociation curve, this expression makes it possible to calculate from the present data the change with pH of the free energy of oxygenation of a single heme group in the hemoglobin molecule.

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## NOTE ON THE QUANTITATIVE PRECIPITATION OF CHOLESTEROL DIGITONIDE IN THE PRESENCE OF BILE SALTS

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While the conditions for the quantitative precipitation of cholesterol digitonide have received considerable attention (1-3), no information appears to be available with regard to the effect of considerable quantities of bile salts. Separation by continuous extraction of the dried bile salt-cholesterol mixture was unsatisfactory. The precipitation of cholesterol digitonide in the presence of bile salts dissolved in alcohol was slow. Errors up to 40 per cent were noted even after the solution had stood overnight.

Quantitative precipitation within the usual limits of error may be obtained by adding a quantity of hydrogen chloride dissolved in alcohol, slightly in excess of the bile salt equivalent,<sup>1</sup> (see Table I).

### EXPERIMENTAL

The effect of the presence of the sodium salts of cholic, desoxycholic, glycocholic (5), glycodesoxycholic (6), taurocholic,<sup>2</sup> and taurodesoxycholic acids was studied. Cholesterol bile salt ratios up to 7:1000 were analyzed.

The general procedure consisted in evaporating a sample of the aqueous cholesterol-bile salt solution containing from 7 to 10

<sup>1</sup> Schoenheimer and Sperry (4) have indicated that small amounts of hydrogen chloride do not interfere with the precipitation of cholesterol digitonide.

<sup>2</sup> The preparation of the "tauro" acids will be described in a subsequent communication.



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mg. of cholesterol to incipient dryness on the steam bath. The residue was dissolved in 10 cc. of boiling 95 per cent alcohol and a 25 to 50 per cent excess of digitonin, dissolved in 90 per cent alcohol, was added. The solution was then brought to a boil and kept at room temperature. At the end of a half hour 1 cc. of normal alcoholic hydrogen chloride was added for each gm. of total solid matter, and an additional quantity of 0.5 cc. to the

TABLE I  
*Recovery of Cholesterol When Added to Various Bile Salts*

| Bile salt present                      | Amount of<br>bile salt | Cholesterol | Digitonide |            | Error    |
|--|------------------------|-------------|------------|------------|----------|
|  |                        |             | Found      | Calculated |          |
|  | gm.                    | mg.         | mg.        | mg.        | per cent |
| Sodium glycocholate                    | 0.1                    | 7.68        | 31.2       | 31.6       | -1.2     |
|  | 0.5                    | 7.68        | 31.3       | 31.6       | 0.95     |
|  | 0.5                    | 7.68        | 30.9       | 31.6       | -2.2     |
|  | 1.0                    | 7.54        | 31.6       | 31.0       | 1.9      |
|  | 1.0                    | 7.54        | 29.8       | 31.0       | 0.64     |
| Sodium glycodes-<br>oxycholate         | 0.2                    | 7.51        | 31.2       | 30.9       | 0.97     |
|  | 0.2                    | 7.51        | 30.7       | 30.9       | -0.65    |
|  | 1.0                    | 7.51        | 30.2       | 30.9       | -2.2     |
|  | 1.0                    | 7.54        | 31.8       | 31.0       | 2.2      |
| Sodium taurocho-<br>late               | 1.0                    | 7.61        | 30.5       | 31.3       | -2.6     |
|  | 1.0                    | 7.61        | 31.0       | 31.3       | -0.93    |
| Sodium taurodes-<br>oxycholate         | 1.0                    | 7.54        | 31.1       | 31.0       | 0.30     |
|  | 1.0                    | 7.54        | 30.7       | 31.0       | -0.97    |
| Sodium cholate                         | 1.0                    | 7.51        | 30.2       | 30.9       | -2.3     |
|  | 1.0                    | 7.51        | 30.5       | 30.9       | -1.3     |
| Sodium desoxycho-<br>late              | 1.0                    | 7.51        | 30.3       | 30.9       | -1.9     |
|  | 1.0                    | 7.51        | 30.3       | 30.9       | -1.9     |
| Equal mixture of 4<br>conjugated salts | 1.0                    | 7.51        | 30.4       | 30.9       | -1.6     |

entire volume of the solution. After standing overnight the precipitate was transferred to a fritted glass filter (Jena No. 1G4) with 7 cc. of 95 per cent alcohol. (A 25 cc. graduated wash bottle with a capillary tip was convenient for this purpose.) The precipitate was washed with 5 cc. of boiling water, then with water at room temperature, until the washings gave a negative test for chloride, and finally with 3 cc. of alcohol in two portions and with ether. It was dried at 105° for 1 hour and weighed.

Owing to the relative insolubility of sodium cholate in alcohol, complete solution occasionally was not obtained until the hydrogen chloride had been added and the residue rubbed with a glass rod. Excess of normal alcoholic hydrogen chloride up to 2.5 cc. did not appreciably affect the completeness of precipitation. Repetition of the entire washing process caused an average loss of 0.3 mg. (about 1 per cent) in twenty determinations.

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## A STUDY OF CERTAIN METABOLITES AND RELATED COMPOUNDS AS PRECURSORS OF ENDOGENOUS CITRIC ACID\*

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The wide distribution of citric acid in common foods (see Hartmann and Hillig (1934)) and its constant presence in small quantities in various tissues and body fluids and in the excreta, particularly the urine, of many mammals, including man (see Thunberg (1929); Östberg (1931); Sherman, Mendel, and Smith (1936, a)) have been adequately demonstrated. There is a considerable amount of evidence, however, which indicates that the citric acid present in the urine does not necessarily arise from the citric acid ingested; in fact, it appears that no more than 2 per cent of the free acid administered orally is excreted in the urine, even when it is given in relatively large amounts (see Kuyper and Mattill (1933); Schuck (1934); Sherman, Mendel, and Smith (1936, b)). On the other hand, if citrate is given as the sodium salt, or if sodium bicarbonate itself is administered, relatively large quantities of citric acid are excreted in the urine (see Östberg (1931); Sherman, Mendel, and Smith (1936, a)). In the dog, the administration of alkali for long periods of time results in the maintenance of the augmented excretion of citric acid, the amount eliminated far exceeding that present in the food ingested (Sherman, Mendel, and Smith, 1936, a). In view of evidence indicating the absence of significant stores of citric acid in different organs and tissues of the dog (Pucher, Sherman, and Vickery, 1936; unpublished data of present authors), the conclusion appears warranted that the

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citric acid was endogenous in origin; undoubtedly, under these conditions, the substance was synthesized in the organism from some precursor or precursors. Evidence of this type has served as a basis for the theory that citric acid serves as one of the "physiological acids" and that it is formed in response to excess alkali in the organism (Östberg, 1931). However, there is further evidence (Kuyper and Mattill, 1933; Boothby and Adams, 1934) which indicates that increases in the citric acid content of the urine may occur irrespective of changes in pH, thus suggesting that citric acid may also be a normal metabolite.

Since it appears that citric acid may be synthesized in the animal body, questions arise regarding the nature of the precursor or precursors of the endogenous substance. Recent work on this problem has yielded rather conflicting results. There is some evidence that either dietary carbohydrate (Sherman, Mendel, and Smith, 1936, *a*; von Fürth *et al.*, 1934), fat, or protein (Boothby and Adams, 1934) may yield metabolites which serve as precursors of citric acid. Also a few studies in the pig have indicated that sodium acetate causes a greater increase in the excretion of urinary citric acid than does an equivalent amount (based on sodium) of sodium bicarbonate, or similar quantities of the lactate or malate, thus suggesting that the acetate radical might serve as a precursor of citric acid (von Fürth *et al.*, 1934). A need for further study of the problem is clearly indicated.

In each of the foregoing investigations, the dietary constituent or the substance to be tested as a precursor was administered orally; the degree of absorption and the influence of bacterial action in the intestine thus become variables of unknown significance in the experiment. Therefore, all compounds used in the present investigation were administered intravenously. Each of the substances chosen for study as a possible precursor of endogenous citric acid was a known, simple metabolite or a closely related compound.

#### EXPERIMENTAL

Four healthy adult female dogs weighing between 15 and 20 kilos and between 1 and 2 years of age were used as experimental subjects. They were kept in individual metabolism cages and fed a synthetic, citrate-low basal ration (Sherman, Mendel, and Smith,

1936, a) having the following composition: casein<sup>1</sup> 37.4 per cent, sucrose 26.4 per cent, lard 16.5 per cent, butter 6.5 per cent, salt mixture (Wesson, 1932) 3.5 per cent, dried yeast<sup>2</sup> 6.8 per cent, bone ash 2.7 per cent, and cod liver oil 0.2 per cent. The quantity of the basal diet fed daily was so adjusted that the animals either maintained a constant body weight near the "ideal" value (Cowgill, 1928) or gained weight slightly. An amount of diet furnishing 60 to 70 calories per kilo of body weight per day was found to be satisfactory in this respect. Analysis of the diet showed that it contained 16.5 to 17.5 mg. of citric acid per 100 gm.

The substances studied as possible citric acid precursors were either the sodium salts of organic acids<sup>3</sup> or a calculated amount of sodium bicarbonate added to the compound under investigation as, for example, glycerol. The sodium salt was prepared in most cases by adding to the c.p. or recrystallized organic acid the theoretical amount of sodium hydroxide. However, because of the extreme alkalinity of solutions of the disodium salts of the amino acids, aspartic and glutamic, and of the sodium salt of glycine, the theoretical amount of sodium bicarbonate was added to sterile solutions of the free acid. The pH of the resultant salts in 5 per cent solutions was approximately 7.5. Some indication of the purity of certain of the compounds employed was obtained from the fact that the theoretical amount of standard alkali used to form the sodium salt sufficed to bring the pH of the aqueous solution of the acid to approximately 7.5. In addition, the purity of all of the organic acids whose sodium salts subsequently yielded a positive response was checked by melting point determinations. The following isomers of the optically active acids were employed: *dl*-lactic acid, *dl*-malic acid, *d*-tartaric acid, *l*-aspartic acid, and *d*-glutamic acid. It is realized, however, that undoubtedly some racemization occurred during the process of preparation and sterilization.

Sterile solutions of the compound under investigation were injected intravenously, under aseptic conditions. A 5 per cent solution of the substance was used, except in the case of sodium

<sup>1</sup> Lister Brothers, New York.

<sup>2</sup> Northwestern Yeast Company, Chicago.

<sup>3</sup> Appreciation is expressed to Dr. G. J. Cox of the Mellon Institute for a generous sample of sodium levulinate.

chloride, which was injected as a 2.5 per cent solution, and glucose and sodium gluconate which were used in 10 per cent solutions. With the exceptions recorded below, the amount of solution injected was adjusted so that 100 mg. of sodium per kilo of body weight were administered. The reason for injecting equivalent amounts of sodium in each instance is the fact that alkali itself exerts a significant effect on the amount of citric acid excreted in the urine. In the case of the butyrate, aspartate, and glutamate, amounts providing 100 mg. of sodium per kilo of body weight were found to produce nausea and vomiting, therefore only one-half the usual amount of these substances was injected. Likewise, only one-half the usual amounts of sodium citrate and maleate was injected because of the toxicity of these compounds. For the sake of comparison of results, one-half doses of sodium malate were also employed.

The citric acid content of aliquots (usually 20 to 25 cc.) of 24 hour urine samples, collected quantitatively and preserved with toluene, was determined by a photometric procedure (Pucher, Sherman, and Vickery, 1936). Hydrogen peroxide was used to remove the excess of potassium permanganate rather than ferrous sulfate. Accurate recoveries and consistent results were obtained with this method; however, it was found essential to pay special attention to the petroleum ether employed, since impurities present in certain samples interfered with the color formation and, as a result, values far too low were obtained. The effect on the method of all the substances found to produce a significant increase in citric acid excretion was determined both in aqueous solution and when added to urine in quantities equal to the maximum amount which could appear in 20 cc. of urine if all the substance were excreted unchanged. With the exception of sodium acetoacetate and one sample of sodium malate, each of the compounds employed gave no color whatsoever in aqueous solution nor added color in the presence of urine. A small amount, approximately 0.1 per cent, of citric acid, or some unknown chromogenic substance, was present in one sample of sodium malate, even after repeated recrystallization of the free acid from water. A trace of added color was produced by sodium acetoacetate when this compound was added to urine. The degree of interference of these two substances, however, was far too small to affect appreciably the results herein

reported. Others (Pucher, Sherman, and Vickery, 1936) have also found that of a number of substances tested, including malic acid, only acetoacetic acid may interfere with the present method for determining citric acid.

The pH of the urine samples was determined routinely in duplicate by a colorimetric procedure, with phenol red and bromthymol blue as indicators. Periodically, qualitative tests for "acetone bodies" and for albumin in the urine were made by standard procedures. The former test was performed because of the indication that acetoacetic acid gives some color when subjected to the procedure used for determining citric acid, and the latter was made to detect possible nephrotoxic action of the materials injected.

At the beginning of the experiment, daily citric acid and pH determinations were made during a preliminary period of 10 days in order to determine the basal citric acid excretion of each animal when fed the unsupplemented diet. During the subsequent experimental periods of 8 days, the compound to be tested was injected daily for the first 3 consecutive days and treatment was suspended for the remaining 5 days; daily citric acid and pH determinations were made during the entire 8 day interval, with the exception of the 7th day. The injections of the various compounds employed were repeated in an intentionally non-systematic order, so that possible after-effects would not be a consistent complicating factor.

### *Results*

Inasmuch as the responses of the four dogs used in these studies were strikingly uniform, only a few data for one typical dog are presented in detail (Fig. 1). Several significant points may be mentioned: the striking constancy of citric acid excretion during the preliminary period when the basal ration alone was administered; the prompt and rather consistent rise in the excretion of citric acid following the administration of sodium malate and certain other compounds; the rapid decrease in citric acid excretion to the basal level following the cessation of daily injections; and, finally, the fact that, although the pH of the urine invariably increased after the injection of most of the compounds employed, the fluctuations did not necessarily correspond in magnitude with



the increases in citric acid excretion. In all of the dogs, albumin was either absent from the urine or no more than a faint trace appeared at any time during the experiment.<sup>4</sup> Acetone bodies appeared in significant amounts only after the injection of sodium acetoacetate; smaller quantities were excreted, however, after the

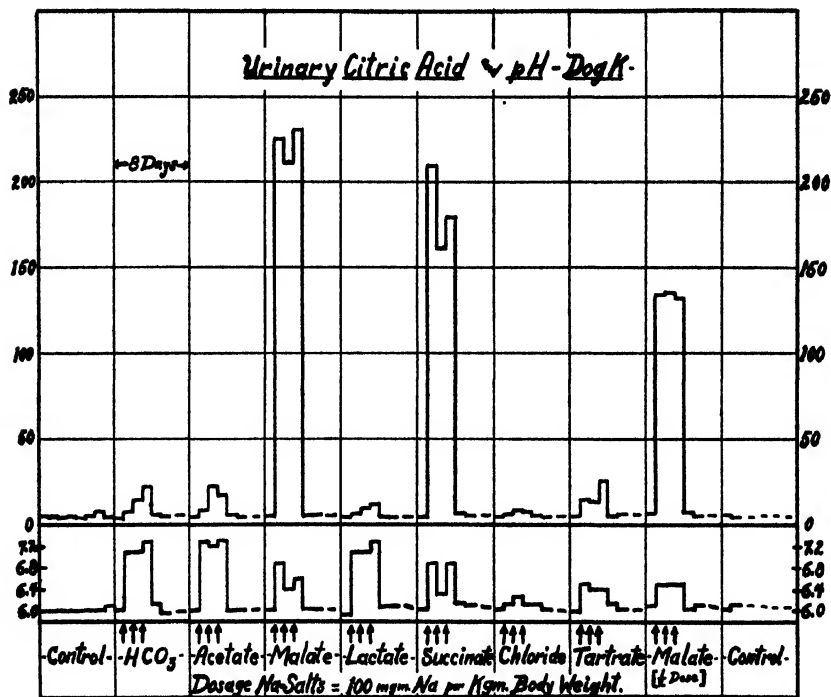


FIG. 1. Changes in the citric acid content and in the pH of 24 hour samples of urine of a typical dog during control periods and following the intravenous injection of certain compounds. The arrows indicate the days on which injections were made.

injection of the malonate, butyrate, levulinate, and, in the one instance, maleate.

<sup>4</sup> The failure of tartrate to exert a nephrotoxic effect under the conditions of the present experiment is undoubtedly due to the fact that the amount of the substance (about 450 mg. of disodium tartrate per kilo of body weight) injected was somewhat less than the minimal quantity found by other investigators (see Underhill *et al.* (1931)) to produce detectable renal damage in the dog.

TABLE I

*Citric Acid Content and pH of 24 Hour Samples of Urine Following Intravenous Injection of Certain Compounds into Dogs*

The results for citric acid are expressed in mg. per kilo of body weight.

| Treatment                   | Citric acid |             | pH      |         | Comment                          |
|-----------------------------|-------------|-------------|---------|---------|----------------------------------|
|                             | Average     | Range       | Average | Range   |                                  |
| Basal ration only           | 0.47        | 0.21- 0.64  | 6.1     | 6.0-6.3 |                                  |
| Sodium chloride             | 0.50        | 0.30- 0.81  | 6.25    | 5.9-6.3 |                                  |
| “ bicarbonate               | 0.90        | 0.44- 1.64  | 7.1     | 6.6-7.3 |                                  |
| “ acetate                   | 1.53        | 0.45- 3.89  | 7.1     | 6.6-7.4 |                                  |
| “ glycolate                 | 0.61        | 0.31- 0.84  | 6.9     | 6.5-7.4 |                                  |
| “ glycinate-bicarbonate     | 1.64        | 0.65- 2.91  | 7.3     | 6.8-7.5 |                                  |
| Glycerol-sodium bicarbonate | 2.01        | 0.66- 4.55  | 7.3     | 6.8-7.6 |                                  |
| Sodium lactate              | 0.73        | 0.36- 1.61  | 7.1     | 6.5-7.3 |                                  |
| “ malonate                  | 14.76       | 10.65-16.78 | 6.6     | 6.4-6.7 |                                  |
| “ butyrate                  | 5.19        | 1.39- 9.08  | 6.9     | 6.4-7.3 | Nausea*                          |
| “ acetoacetate              | 3.50        | 0.85- 8.66  | 7.2     | 6.6-7.4 |                                  |
| “ succinate                 | 14.80       | 11.30-16.30 | 6.6     | 6.3-6.9 |                                  |
| “ fumarate                  | 17.10       | 12.78-21.69 | 6.6     | 6.3-6.9 |                                  |
| “ maleate                   | 61.30       |             | 6.6     |         | Toxic.*<br>One dog only          |
| “ malate                    | 18.50       | 8.76-26.13  | 6.6     | 6.4-6.9 |                                  |
| “ malate-bicarbonate        | 18.80       | 17.18-21.04 | 6.6     | 6.2-7.0 | *                                |
| “ tartrate                  | 1.97        | 0.68- 4.44  | 6.4     | 6.2-6.5 |                                  |
| “ aspartate-bicarbonate     | 3.96        | 1.20- 7.78  | 6.6     | 6.3-7.6 | Nausea*                          |
| “ levulinate                | 3.46        | 1.00-10.14  | 6.8     | 6.6-6.9 | Three dogs                       |
| “ glutamate-bicarbonate     | 1.56        | 0.79- 2.59  | 6.8     | 6.5-7.2 | Nausea*                          |
| “ gluconate                 | 0.41        | 0.19- 0.74  | 6.3     | 6.1-6.5 |                                  |
| “ citrate                   | 115.2       |             | 6.1     |         | Slightly toxic.*<br>One dog only |
| Glucose-sodium bicarbonate  | 0.74        | 0.55- 1.13  | 7.2     | 6.8-7.5 | Two dogs only                    |

\* Injected in amounts supplying 50 mg. of sodium per kilo of body weight; the data given for these compounds, however, are twice the actual values obtained, so that they are comparable with those given for each of the other substances, which were injected in amounts supplying 100 mg. of sodium per kilo of body weight.

The average daily excretion of citric acid for the four dogs, expressed as mg. per kilo of body weight, and the average urinary pH, together with the actual range of variation of the values, obtained with each substance tested are given in Table I. It is evident from these data that the compounds employed may be grouped arbitrarily into four classes according to their effect on the excretion of citric acid. The first group of substances, which includes sodium chloride and gluconate, does not appear to exert a significant effect on the excretion of citric acid. The second group of compounds, sodium bicarbonate, acetate, glycolate, glycinate-bicarbonate, lactate, glycerol-bicarbonate, tartrate, glutamate-bicarbonate, and glucose-bicarbonate, appear to exert an "alkali" effect only, the citric acid output being increased 2 to 4 times the basal value. The third group of compounds, sodium butyrate, acetoacetate, aspartate-bicarbonate, and levulinate, appears to stimulate an excretion of citric acid distinctly greater than the typical alkali response, the amounts varying from 7 to 10 times those excreted during the period in which the basal ration alone is given. The final group of compounds, sodium malonate, succinate, fumarate, malate, and maleate, elicited a marked increase in citric acid excretion, the values obtained after the injection of these substances being 30 to 40 times greater in the case of the first four and 120 times greater in the case of the single injection of maleate than the basal output. Particularly significant is the fact that the injection of the half dose (50 mg. of sodium per kilo of body weight) of sodium malate uniformly elicited approximately half the increase in citric acid that was obtained with the full dose (100 mg. of sodium per kilo of body weight). Approximately 40 per cent of the sodium citrate injected intravenously into the one animal was excreted in the urine. A similar degree of destruction of sodium citrate following its intravenous injection has been reported by others (Salant and Wise, 1916-17).

Likewise, the results show a general tendency to group themselves with reference to the influence on pH of the 24 hour urine samples. The chloride and gluconate caused only a slight or questionable rise in pH, whereas marked rises followed the injection of bicarbonate and the majority of the other compounds. However, it is significant that the pH of the urine following the injection of the five substances most effective in increasing the output

of citric acid was intermediate between that found during control periods and that obtained after the injection of sodium bicarbonate and the other compounds exerting an "alkali" effect. This is another instance in which the excretion of citric acid in the urine does not parallel the pH. The failure of injected citrate, in the one animal, to affect the pH of the urine materially should be mentioned in this connection.

Inasmuch as the effect of maleate was studied in only one animal, the results should be considered only in a tentative sense. Because of the magnitude of the rise elicited by this substance, however, there is little doubt of its importance as a precursor of citric acid. The administration of maleate was limited to a single injection because of its extreme toxicity. Even in the amount used, toxic symptoms slowly developed, followed by the death of the animal 72 hours after injection.<sup>5</sup>

With the exception of the maleate and citrate, no evidence of definite toxicity of the amounts of the salts employed was observed. However, marked nausea and vomiting followed the injection of certain substances; namely, the aspartate, butyrate, and glutamate.

#### DISCUSSION

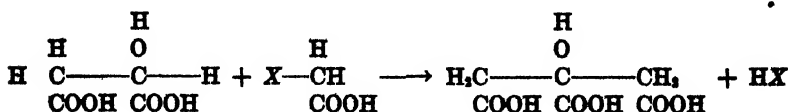
It is evident from the present data that under the conditions employed the injection of the disodium salts of malonic, succinic, fumaric, malic, and probably maleic acids is followed by a marked increase in the urinary excretion of citric acid. There are several reasons for believing that these compounds do not stimulate the production of citric acid in some indirect manner, but that they actually enter into its formation and, in that sense, are true precursors of endogenous citric acid. In view of the evidence indicating a lack of significant stores of citric acid in various body fluids and tissues, it does not seem probable that the effect of the injected substances is a liberation of the preformed compound. Nor does there appear to be a justification for assuming an impairment of a "citrate-destroying mechanism" with a subsequent increased urinary excretion of the substance, particularly in view of the uniform, almost stoichiometric relation in the case of maleate

<sup>5</sup> Appreciation is expressed to Dr. A. W. Workman of the Department of Bacteriology for performing the autopsy and for advice regarding the findings.

between the amount of substance injected and the quantity of citric acid appearing in the urine. The most tenable explanation appears to be that the five most effective compounds themselves, or some product formed from them in the organism, actively enter into the formation of citric acid. Likewise, the finding that certain compounds, those in the foregoing third group, elicit a greater increase in citric acid excretion than can be attributed to an "alkali" effect, can be logically explained by postulating that relatively small amounts of these substances are converted in the organism into degradation products which are similar to those derived from the five most potent precursors.

Of the effective 4-carbon atom dicarboxylic acids, exclusive of maleic acid, malic acid uniformly elicited a somewhat greater excretion of citric acid than did the others. This finding may be interpreted as indicating that succinic and fumaric acids must be converted into malic acid, a process entailing some loss, before they are synthesized into citric acid. In this connection, it is significant that tartaric acid, in contrast to malic acid, elicited little, if any more of an increase in citric acid excretion than did sodium bicarbonate. As further evidence of a close connection between the behavior of citric and malic acids in living matter, may be mentioned the frequent inverse relationship between the amounts of these substances present in certain fruits and vegetables (Hartmann and Hillig, 1934) and in the growing tobacco plant (Vickery *et al.*, 1935). On the other hand, it has been reported (von Fürth *et al.*, 1934) that 50 gm. of sodium malate administered orally to a pig produced no significant increase in citric acid excretion in the urine. Since only one animal was used, however, this experiment should be repeated before final conclusions regarding the ineffectiveness of malate given by mouth are drawn.

It is not difficult to formulate simple reactions by which citric acid can be formed from 4-carbon atom, dicarboxylic acids, especially malic acid. For example, the following suggestion (see von Fürth *et al.* (1934)) has been made.



The conversion of succinic, fumaric, and maleic acids into malic acid could easily occur, and compounds with 2-carbon atoms such as glycine, glycolic, or even acetic acid are undoubtedly readily available in the organism. Also, the suggestion has recently been made, based on experiments *in vitro* (Knoop and Martius, 1936), that citric acid is easily formed from oxalacetic acid and pyruvic acid. As far as the authors are aware, however, no experimental evidence of such conversions *in vivo* has thus far been described.\*

That the story of the formation of citric acid in the organism is not quite so simple as the above discussion might lead one to believe is indicated by the fact that the increase in citric acid excretion following the injection of sodium malonate was nearly as great as that found after the injection of sodium malate. It is difficult to indicate a simple reaction showing the conversion of malonic to citric acid; possibly 3 molecules of malonic acid upon oxidation and decarboxylation form 1 molecule of citric acid. In view of the findings with malonic acid, the possibility that citric acid may be formed from the effective 4-carbon atom, dicarboxylic acids only after the degradation of these compounds into 3-carbon atom, or perhaps even 2-carbon atom residues must be considered. A final solution of the problem obviously awaits further studies on the metabolism of the compounds involved.

Questions as to the origin in the animal organism of the precursors under discussion may be raised. It is possible that they may be exogenous in origin, since certain of these substances, particularly malic acid, are widely distributed in various foods (Hartmann and Hillig, 1934). Also there is evidence that malic acid as well as fumaric and succinic acids may be formed in muscle during the metabolic degradation of aspartic and glutamic acids (Needham, 1930). Other investigators have repeatedly described the presence of malic, succinic, and fumaric acids in certain animal tissues (see Dakin (1922); Needham (1930)) and, indeed, they have been suggested as important intermediary compounds in the process of tissue respiration (Annau *et al.*, 1935).

The fact that sodium malate and fumarate were highly effective

\* It is recognized, however, that certain of the foregoing organic acids may be involved in the production of citric acid by fungi (see Iwanoff and Zwetkoff (1936)).

in promoting an increase in citric acid excretion might lead one to assume that aspartic acid would exert a similar effect, and that endogenous citric acid was formed from the aspartic acid derived from dietary or tissue protein. However, the finding that aspartic acid stimulated a much smaller increase in citric acid excretion than did malic acid lends little support to such a view. On the other hand, the present data do not necessarily preclude the possibility that aspartic acid derived from protein is an important precursor of endogenous citric acid. It may be that the deamination of the injected aspartic acid is a very slow process, or that it may follow several different paths of metabolism, or perhaps that considerable amounts of the unchanged substance are rapidly excreted. It is also conceivable that the effective 4-carbon atom, dicarboxylic acids under discussion might be formed during the metabolic degradation of carbohydrates or fats (see Needham (1930)). A further elucidation of this question will undoubtedly follow subsequent studies on the metabolic origin of the substances malonic, succinic, fumaric, and malic acids.

#### SUMMARY

A number of common metabolites and related substances were studied as possible precursors of citric acid by determining their effect after intravenous injection on the daily excretion of citric acid in the urine of dogs fed a constant amount of a citrate-low basal diet.

Of the many compounds tested, the disodium salts of malonic, succinic, fumaric, malic, and, in one animal, maleic acids were found to stimulate by far the greatest increase in citric acid excretion, even though they produced only moderate increases in urinary pH.

In view of evidence indicating the absence of significant stores of preformed citric acid in the dog and a rather specific relationship between the structure and dosage of the injected substances and the effect elicited, the conclusion appears warranted that these dicarboxylic acids or their metabolic degradation products actively enter into the formation of citric acid in this species and, in that sense, are precursors of endogenous citric acid.

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## THE $pK'$ OF SERUM AND RED CELLS

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The pH of serum is often calculated from free and combined  $CO_2$  by use of the Henderson-Hasselbalch equation

$$pH = pK' + \log \frac{BHCO_2}{H_2CO_2}$$

In this equation  $BHCO_2$  represents the difference between total  $CO_2$  and dissolved  $CO_2$ ,  $H_2CO_2$ . The value for  $pK'$  in serum,  $pK'_s$ , is accurately established; our measurements were made to see whether this function varies over the extreme physiological range of pH as well as to give assurance of the reliability of technique.

The  $pK$  in red cells,  $pK'_e$ , is not necessarily the same as in serum, since, for one reason, a considerable fraction of the  $CO_2$  of red cells is combined with hemoglobin. The proportion of this compound, carbhemoglobin, depends on degree of oxygenation and on the pH, and hence  $pK'_e$  may depend on the same functions. Indeed the measurements of Stadie and Hawes (1) led them to the conclusion that in reduced cells  $pK'_e$  is greater by 0.11 than in oxygenated cells. Their measurements were made before Van Slyke, Sendroy, Hastings, and Neill (2) determined the solubility coefficient of  $CO_2$  in hemoglobin solutions. When their coefficient is used in recalculating the data of Stadie and Hawes, the  $pK'_e$  values of reduced cells range from 5.93<sup>1</sup> to 6.07 and of cells satu-

<sup>1</sup> The value 5.93 may be wrong, for there is an inconsistency in tabulated values of  $\alpha_{M.F.}$  and pH (see (1), the last line of Table XV). According to our recalculations,  $pK'_e$  values are increased by 0.04 in the data of Van Slyke, Hastings, Murray, and Sendroy (3). With one exception the increase is from 0.08 to 0.10 in the  $pK'_e$  values given by Stadie and Hawes (1). The greater magnitude of the correction is related to their assumption that all their solutions had a density of unity (see their foot-note on p. 274).

rated with CO from 6.10 to 6.12. The values of Van Slyke, Hastings, Murray, and Sendroy (3) in reduced cells range, after recalculation, from 5.87 to 6.03, averaging 5.97.

It is a curious fact that although the temperature of man is 37°, temperatures of 37.5° or 38° are usually employed in determining pH of human blood. Under some circumstances the difference is unimportant; under others, a matter of considerable importance. For example, in studying gas equilibria in the lungs a difference of 1° in the equilibration temperature may alter the estimated partial pressure of CO<sub>2</sub> in the blood more than 2 mm. Blood which will show  $\Delta p\text{CO}_2$  between lungs and air of 1 mm. when equilibrated at 37° will show  $\Delta p\text{CO}_2$  of 3.4 mm. at 38°. If the first value is correct, the second value will be in error by 240 per cent. Accordingly, since our study of 1929 on gas equilibria (4), we have employed either a temperature of 37° or the observed body temperature of the subject. Other illustrations will come to mind in which significant errors are introduced by the employment of a temperature in the study of human blood which is not that of the body.

The *solubility coefficient for CO<sub>2</sub> in serum* at 38° has been determined by Van Slyke, Sendroy, Hastings, and Neill (2). According to them

$$\begin{array}{lcl} \text{mm (H}_2\text{CO}_3) \text{ per kilo serum water} & = & 0.0327 \text{ } p\text{CO}_2 \\ \text{" " " " cell " "} & = & 0.0354 \text{ "} \end{array}$$

It may be assumed that the temperature effect on these solubility factors is the same as on  $\alpha_{\text{CO}_2}$  in water. Accordingly at 37°,

$$\begin{array}{lcl} \text{mm (H}_2\text{CO}_3) \text{ per kilo serum water} & = & 0.0334 \text{ } p\text{CO}_2 \\ \text{" " " " cell " "} & = & 0.0362 \text{ "} \end{array}$$

These are the factors we have employed.

*Serum pK'*—The essential features of the method will be described below. The values found in four experiments on sera of three mammals are shown graphically in Fig. 1. There is some evidence of increase in pK', with increasing acidity, although this cannot be considered conclusive. The mean value of all determinations, 6.112, is in excellent agreement with the data assembled by Hastings, Sendroy, and Van Slyke (5). Their mean value for ten non-nephritic sera calculated to 37°, assuming a temperature coefficient of  $-0.005$ , is 6.110 and for a series including these and

six nephritics, 6.116. We conclude, therefore, that the  $pK'$  of mammalian serum at  $37^\circ$  is 6.11 in the usual physiological range.<sup>3</sup>

*Cell  $pK'$* —Eight experiments have been carried out to determine

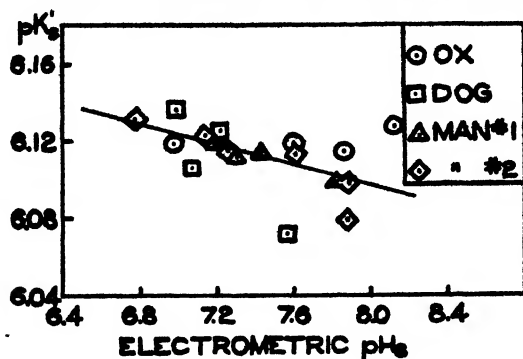


FIG. 1. The  $pK'$  of mammalian sera at  $37^\circ$ . The mean value is 6.112 and the mean deviation from this value is 0.010. With the exception of a single widely divergent value, all points obtained within  $pCO_2$  values of 7 and 220 mm. are included. The evidence for dependence of  $pK'$  on  $pH$  is best in human serum. The slope,  $-0.03$ , is only one-fifth that found in cells (see equations on p. 577).

TABLE I  
*Properties of Cell Hemolysates*

| Experiment No. | Species | Total Hb  | Solids             | Sp.gr. | H <sub>2</sub> O | Serum              | CO <sub>2</sub> solubility factor |
|----------------|---------|-----------|--------------------|--------|------------------|--------------------|-----------------------------------|
|                |         | mm per l. | per cent by weight |        | gm. per l.       | per cent by volume |                                   |
| 1              | Ox      | 20.2      | 35.2               | 1.105  | 717              | 0                  | 0.0260                            |
| 2              | "       | 16.6      | 29.0               | 1.085  | 771              | 23                 | 0.0273                            |
| 3              | "       | 18.9      | 33.3               | 1.097  | 743              | 10                 | 0.0268                            |
| 4              | Dog     | 18.6      | 31.3               | 1.094  | 752              | 14                 | 0.0268                            |
| 5              | Man     | 19.6      | 33.6               | 1.101  | 730              | 4                  | 0.0262                            |
| 6              | Ox      | 18.3      | 31.4               | 1.094  | 751              | 14                 | 0.0268                            |
| 7              | Man     | 19.6      | 34.3               | 1.103  | 724              | 2                  | 0.0261                            |
| 8              | Ox      | 20.4      | 34.0               | 1.102  | 726              | 2                  | 0.0261                            |

the  $pK'$  of oxygenated and reduced red cells. Five of these were with ox cells, one with dog cells, and two with human cells. In the

<sup>3</sup>The values found by Robinson, Price, and Cullen, (6) are consistently below 6.11. However, the mean of the three sets of observations on human serum at  $37^\circ$  is 6.108.

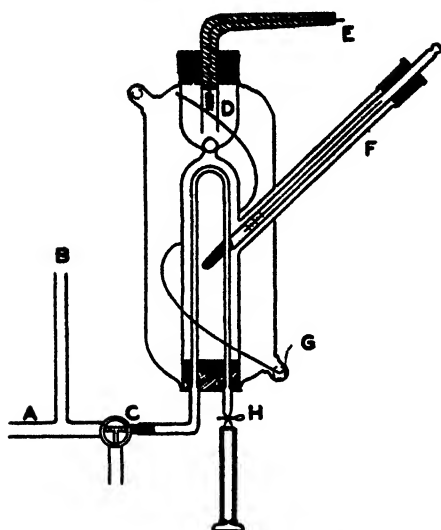


FIG. 2. The electrode proper, of Corning glass No. 015, is a U-shaped tube with a wall less than 0.1 mm. in thickness. With a length of 200 mm. and inside diameter of 1.5 mm., the volume is about 0.40 cc. The tube is bent by its own weight while a positive pressure is maintained within the tube and the mid-portion heated gently with a small luminous flame. Then the wall of the tube is thinned by thoroughly cleaning and dipping in hydrofluoric acid. The process is controlled by frequently removing the electrode, rinsing in water, and then observing the change in the amount of pressure required to bend the tube. A satisfactory electrode will give a potential of approximately 0.0615 volt per unit change in pH at 37°. The electrode is held in position between the halves of a rubber stopper which is sealed and fastened together with thick rubber cement and a wire band. 0.1 N HCl surrounds the electrode in the inner chamber and fills the side tube *F*. An Ag-AgCl electrode, connected to an amplifier at *E*, is placed within a paraffined tube at *D*, and a large glass bead is placed above the constriction in order to minimize movements of solution around the Ag-AgCl electrode. Water from a constant temperature bath maintains the temperature by circulating in the outer chamber. We have used the electrode successfully at 0.5°, 25°, and 37°. A small copper wire is grounded at *G* and passed through the rubber tubing and connection into the outer chamber in order to remove stray electric currents which might result from the circulating water.

The sample is introduced into the electrode at *H* with a 2 cc. syringe and a few drops allowed to escape through the stop-cock at *C*, using only 0.7 or 0.8 cc., then a small clip is placed on the rubber tube at *H* and the cock at *C* partly turned. After 2 or 3 minutes equilibrium is reached in the electrode and the cock (*C*) is turned so as to make connection with the calomel

first two experiments, ox cells were obtained by centrifuging freshly drawn, defibrinated blood. The cells were washed twice with approximately isotonic NaCl solution, reduced with nitrogen, and hemolyzed by freezing and thawing several times. Enough dry  $\text{NaHCO}_3$  was added to restore the available base to its normal level. In subsequent experiments washing was dispensed with and addition of bicarbonate was not required. Some serum was present but proper correction was made for its effect on solubility of  $\text{CO}_2$ . The properties of the solutions used are shown in Table I.

Equilibration was carried out for half an hour in tonometers of the original Barcroft type. Experiment showed that with 8 cc. of hemolysate in the tonometer equilibrium was complete in this time, even with very high or low concentrations of  $\text{CO}_2$  in the gas phase. The hemolysate was then transferred in the bath into tubes by displacement of mercury. By using the same pipette throughout a given experiment, it was found that satisfactory duplicates on the Van Slyke apparatus could be obtained.<sup>3</sup> The actual volume delivered was determined by transferring duplicate samples into tared glass-stoppered dishes containing a piece of filter paper for cleaning the tip of the pipette. The weighed samples were dried to constant weight and the specific gravity

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electrode through the salt bridge (A). The potential is determined by means of a potentiometer in the usual way. A duplicate determination is made, without rinsing, by forcing out the solution in the electrode with a fresh portion of the sample and proceeding as before. Between each pair of determinations the electrode is rinsed out several times with distilled water and the last portion allowed to remain in the electrode for several minutes. The best results are obtained when both rinse water and sample are brought to the approximate temperature of the electrode just before being introduced. Cock C is flushed with KCl from a reservoir at B.

If difficulty is experienced in bending the thin glass tube, one can make the electrode straight. The inner chamber of the jacketed vessel is made smaller, without a constriction, the glass electrode is held in position by a rubber stopper at each end, and the sample introduced at the top. In this case the Ag-AgCl electrode is placed in the side tube along with the thermometer.

<sup>3</sup> The only exception was with the hemolysate of dog cells. This was so viscous that the addition of one-tenth its volume of isotonic saline was necessary.

TABLE II

*Data on pK' of Oxygenated and Reduced Cells*

| Experiment 1. Oz cells               |      |       |                  |      |       |                              |
|--------------------------------------|------|-------|------------------|------|-------|------------------------------|
| Oxygenated                           |      |       | Reduced          |      |       | Per cent<br>HbO <sub>2</sub> |
| pCO <sub>2</sub>                     | pH   | pK'   | pCO <sub>2</sub> | pH   | pK'   |                              |
| 15.0                                 | 7.33 | 6.042 | 12.4             | 7.45 | 5.995 | 3                            |
| 19.5                                 | 7.30 | 6.052 | 17.5             | 7.40 | 5.998 | 3                            |
| 37.3                                 | 7.22 | 6.053 | 30.0             | 7.33 | 6.005 | 2                            |
| 54.2                                 | 7.16 | 6.057 | 50.7             | 7.22 | 6.022 | 3                            |
| 95.5                                 | 7.05 | 6.062 | 106.0            | 7.07 | 6.030 | 2                            |
| 195.0                                | 6.89 | 6.058 | 184.0            | 6.93 | 6.024 | 3                            |
| Mean...                              | 7.16 | 6.054 |                  | 7.23 | 6.013 | 3                            |
| $\Delta pK' = pK'_o - pK'_r = 0.041$ |      |       |                  |      |       |                              |
| Experiment 2. Oz cells               |      |       |                  |      |       |                              |
| 4.9                                  | 7.38 | 6.124 | 4.1              | 7.52 | 6.032 | 4                            |
| 14.0                                 | 7.30 | 6.111 | 11.7             | 7.41 | 6.015 | 3                            |
| 34.5                                 | 7.18 | 6.112 | 36.6             | 7.24 | 6.050 | 3                            |
| 117.7                                | 6.96 | 6.124 | 112.3            | 7.00 | 6.067 | 3                            |
| Mean...                              | 7.11 | 6.118 |                  | 7.29 | 6.041 | 3                            |
| $\Delta pK' = 0.059$                 |      |       |                  |      |       |                              |
| Experiment 3. Oz cells               |      |       |                  |      |       |                              |
| 5.8                                  | 7.51 | 6.104 | 5.0              | 7.62 | 5.971 | 3                            |
| 12.7                                 | 7.45 | 6.098 | 10.1             | 7.55 | 5.974 | 3                            |
| 25.2                                 | 7.33 | 6.090 | 21.4             | 7.44 | 6.027 | 2                            |
| 53.2                                 | 7.21 | 6.106 | 49.3             | 7.28 | 6.027 | 2                            |
| 132.8                                | 7.01 | 6.097 | 135.7            | 7.03 | 6.039 | 2                            |
| Mean...                              | 7.30 | 6.099 |                  | 7.38 | 6.008 | 2                            |
| $\Delta pK' = 0.079$                 |      |       |                  |      |       |                              |
| Experiment 4. Dog cells              |      |       |                  |      |       |                              |
|                                      |      |       | 8.5              | 7.35 | 5.997 | 2                            |
|                                      |      |       | 39.9             | 7.14 | 6.030 | 2                            |
|                                      |      |       | 83.3             | 7.01 | 6.055 | 2                            |
|                                      |      |       | 162.0            | 6.86 | 6.063 | 2                            |
| Mean.....                            |      |       |                  | 7.09 | 6.036 | 2                            |

TABLE II—*Concluded*

| Experiment 5. Human cells |      |       |                           |      |       |                              |
|---------------------------|------|-------|---------------------------|------|-------|------------------------------|
| Oxygenated                |      |       | Reduced                   |      |       | Per cent<br>HbO <sub>1</sub> |
| pCO <sub>2</sub>          | pH   | pK'   | pCO <sub>2</sub>          | pH   | pK'   |                              |
| 5.1                       | 7.27 | 6.013 | 4.0                       | 7.42 | 5.921 | 3                            |
| 10.5                      | 7.23 | 6.029 | 8.7                       | 7.38 | 5.964 | 3                            |
| 25.4                      | 7.17 | 6.070 | 23.8                      | 7.28 | 6.016 | 3                            |
| 52.8                      | 7.07 | 6.071 | 48.4                      | 7.17 | 6.018 | 3                            |
| 91.0                      | 7.01 | 6.097 | 121.0                     | 6.99 | 6.024 | 2                            |
| Mean...                   | 7.15 | 6.056 |                           | 7.25 | 5.989 | 3                            |
| $\Delta pK' = 0.067$      |      |       |                           |      |       |                              |
| Experiment 6. Ox cells    |      |       | Experiment 7. Human cells |      |       |                              |
|                           |      |       |                           |      |       |                              |
| 7.7                       | 7.42 | 6.051 | 7.1                       | 7.29 | 5.923 |                              |
| 14.5                      | 7.35 | 6.050 | 9.5                       | 7.28 | 5.944 |                              |
| 19.2                      | 7.30 | 6.073 | 14.1                      | 7.25 | 5.967 |                              |
| 36.9                      | 7.23 | 6.084 | 26.1                      | 7.18 | 5.971 |                              |
| 46.2                      | 7.19 | 6.079 | 111.3                     | 6.96 | 6.022 |                              |
| 73.1                      | 7.11 | 6.087 | 220.0                     | 6.81 | 6.029 |                              |
| 76.5                      | 7.08 | 6.075 |                           |      |       |                              |
| 143.3                     | 6.94 | 6.068 |                           |      |       |                              |
| 200.4                     | 6.86 | 6.073 |                           |      |       |                              |
| Mean...                   | 7.16 | 6.071 |                           | 7.11 | 5.976 |                              |
| Experiment 8. Ox cells    |      |       |                           |      |       |                              |
|                           |      |       |                           |      |       |                              |
| 7.6                       | 7.49 | 6.050 | 10.4                      | 7.54 | 5.977 |                              |
| 12.6                      | 7.43 | 6.061 | 13.7                      | 7.50 | 5.987 |                              |
| 16.2                      | 7.40 | 6.068 | 15.0                      | 7.49 | 5.998 |                              |
| 27.8                      | 7.33 | 6.080 | 19.2                      | 7.46 | 6.006 |                              |
| 42.9                      | 7.24 | 6.074 | 25.5                      | 7.39 | 5.995 |                              |
| 77.6                      | 7.13 | 6.083 | 37.7                      | 7.33 | 6.020 |                              |
| 165.0                     | 6.97 | 6.096 | 69.5                      | 7.20 | 6.028 |                              |
|                           |      |       | 95.9                      | 7.12 | 6.023 |                              |
|                           |      |       | 133.1                     | 7.04 | 6.025 |                              |
| Mean...                   | 7.28 | 6.073 |                           | 7.34 | 6.010 |                              |
| $\Delta pK' = 0.063$      |      |       |                           |      |       |                              |

estimated by the relation  $\text{sp. gr.} = 1.3 - 0.3 (\text{H}_2\text{O})$ , where  $(\text{H}_2\text{O}) = \text{gm. of water per gm. of solution}$ .

About 5 cc. of equilibrated hemolysate served for duplicate gas



analyses on the Van Slyke apparatus and for determination of pH on the glass electrode. The electrode used has been designed by one of the authors (C. D.), and, since it combines the advantages of ruggedness, high sensitivity, and ease of manipulation, a sketch with a description of essential features is shown in Fig. 2. Only 1.5 cc. of solution are required for a pH determination in duplicate. The electrode was standardized several times during each series of pH determinations by means of three phosphate buffer solutions having pH values of 6.788, 7.365, and 7.701 at 37°. These pH values were assigned by Hastings and Sendroy (7), using a hydrogen electrode with 0.1 N HCl (pH 1.08) as a standard of reference, and checked by us, using similar technique.

The partial pressure of CO<sub>2</sub> in the gas phase of the tonometers was determined by analysis on the Haldane apparatus. In calculating (H<sub>2</sub>CO<sub>3</sub>) from *p*CO<sub>2</sub>, account was taken of the presence of serum. It was assumed that cells free from serum contain 720 gm. of H<sub>2</sub>O per liter and that serum contains 940 gm. of H<sub>2</sub>O per liter. From the determined value of H<sub>2</sub>O in the hemolysate the proportion of cells was estimated. A properly weighted solubility factor was derived from this proportion and from the factors given above for solubility of CO<sub>2</sub> in serum and in cells at 37°.

All essential observations are presented in Table II. Every determination is included that falls within *p*CO<sub>2</sub> limits of 4.1 and 220 mm. Outside this range the experimental error with our method is increased. Values for H<sub>2</sub>CO<sub>3</sub> and BHCO<sub>3</sub> are omitted to save space but they can be derived from data given in Tables I and II if desired.

An examination of the data in Table II indicates that in both the oxygenated and reduced states pK', in ox cells at a given pH is greater by about 0.04 than in human cells. There also appears to be some variation in the level of pK' in different hemolysates of ox cells, Experiment 2 giving notably high results. Our technique in handling the viscous hemolysate improved with experience. The last experiment on ox cells appears to be the best; if one plots pK', as a function of pH, the difference between the reduced and the oxygenated state is sharply defined and the decrease in pK', with increasing pH is equally evident. For the oxygenated state, the mean deviation of pK', values from the best fitting straight line is 0.005; for the reduced state, 0.008. This is as satisfactory

a result as one ordinarily gets with serum. If one plots all the results on ox cells together, the scatter of points is greater but the best fitting straight lines fit the points of Experiment 8 very closely.

The results with human hemolysate are not as satisfactory, probably because crystallization occurs in the more acid oxygenated hemolysate. The best fitting straight line for relating  $pK'$  to  $pH_o$  in the reduced state corresponds to the equation  $pK' = 7.275 - 0.18 pH_o$ .

Assuming that the effects of oxygenation on  $pK'$  in man and in the ox are equal, a corresponding equation for the oxygenated state is  $pK' = 7.12 - 0.15 pH_o$ . The mean deviation of observed  $pK'$  values from the line corresponding to this equation is 0.020.

These equations should not be applied outside the range of  $pH$  and of  $pCO_2$  covered by our experiments. It has been shown by Dill, Forbes, and Henderson (8) that in solutions of horse hemoglobin with low  $CO_2$  pressures or with low available base the relation between  $pK'$  and  $pH$  is far from linear.

While our method reveals a distinct trend of  $pK'$  with  $pH$  in both oxygenated and reduced cells, under ordinary conditions this may be neglected. In the usual physiological range of human blood we may assume that  $pK'$  in the reduced state is 5.98 and in the oxygenated state, 6.04. The former is only 0.01 higher than the value recalculated from the data of Van Slyke, Hastings, Murray, and Sendroy (3). The effect of oxygenation on  $pK'$ , however, appears to be about one-half that derived by Stadie and Hawes (1).

These results will be applied at a later time to a description of the  $pH_o$  changes in the respiratory cycle in man in various states. They also may be used for a more precise estimate of the distribution of the hydrogen ion between cells and serum in relation to  $pH$  and degree of oxygenation. They should facilitate the calculation of the proportion of non-bicarbonate  $CO_2$  in the blood. While it is not readily possible to make a quantitative comparison of our results with those of Margaria and Green (9), who employed crystalline horse hemoglobin with added electrolytes and water, the trend of  $pK'$  with  $pH$  revealed by their experiments appears to be of the same order of magnitude as that we have observed in hemolyzed cells.

*Effect of Temperature on  $pK'$  and  $pH$* —An experiment carried

out on oxygenated hemolysate of ox cells is shown in Table III. The effect of temperature on pK' is the same as that found by Cullen, Keeler, and Robinson (10) on serum; *viz.*,  $-0.005$  unit for a rise of  $1^\circ$ . The effect of temperature on pH<sub>a</sub> appears to increase with pH. At pH<sub>a</sub> 7.0 at  $37^\circ$   $\Delta\text{pH}/\Delta t = -0.014$ , while at pH<sub>a</sub> 7.3 the ratio is  $-0.017$ . An experiment carried out on reduced hemolysate of human cells gave exactly the same values.

The temperature coefficient for pH<sub>a</sub> also appears to vary with the reaction. At pH<sub>a</sub> 6.6  $\Delta\text{pH}/\Delta t = -0.007$ , while at pH<sub>a</sub> 7.8 the ratio is  $-0.017$ .

TABLE III

*Experiment 6-a. Effect of Temperature on pH and pK of Oxygenated Cell Hemolysate*

The hemolysate used was that of Experiment 6.

| Equilibration temperature | pCO <sub>2</sub> | H <sub>2</sub> CO <sub>3</sub> * | BHCO <sub>3</sub> | pH at 25° | pH at 37° | $\frac{\Delta\text{pH}}{\Delta t}$ | pK'    |        |
|---------------------------|------------------|----------------------------------|-------------------|-----------|-----------|------------------------------------|--------|--------|
|                           |                  |                                  |                   |           |           |                                    | At 25° | At 37° |
| °C.                       | mm. Hg           | mm per l.                        | mm per l.         |           |           |                                    |        |        |
| 37                        | 19.2             | 0.514                            | 9.00              | 7.524     | 7.315     | 0.174                              |        | 6.072  |
| 37                        | 76.5             | 2.05                             | 21.17             | 7.273     | 7.090     | 0.153                              |        | 6.074  |
| 25                        | 18.3             | 0.659                            | 12.99             | 7.423     | 7.225     | 0.165                              | 6.129  |        |
| 25                        | 71.1             | 2.56                             | 26.48             | 7.157     | 6.985     | 0.143                              | 6.143  |        |

Mean  $\Delta\text{pK}'_a/\Delta t = 0.005$

\* The CO<sub>2</sub> factor at  $37^\circ$  is given in Table I. In calculating the factor at  $25^\circ$  it is assumed that the effect of temperature is the same as in water. This gives a value of 0.0360.

#### SUMMARY

The pK' of serum of man, ox, and dog at  $37^\circ$  is 6.11, a confirmation of earlier data in the literature.

Equations have been derived for the relation of pK' to pH in human cells, but for ordinary purposes pK' in the oxygenated state may be taken as 6.04 and in the reduced state, 5.98.

The pK' of ox cells is variable but in most cases was greater than that of human cells. The effects of pH and of oxygenation on pK' were of the same order of magnitude in the two species.

The temperature coefficient for pK' of cell hemolysate is the same as for serum; *viz.*,  $-0.005$  for a rise of  $1^\circ$ .

The temperature coefficient for  $\text{pH}_i$  increases with  $\text{pH}$  but it is approximately  $-0.016$  for a rise of  $1^\circ$  at the ordinary reaction of red cells in arterial blood.

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## THE RELATION OF GLYCINE AND SERINE TO GROWTH\*

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The isolation of the new amino acid, threonine, has rendered it possible to induce growth in animals by the use of synthetic diets in which the proteins are replaced by mixtures of highly purified amino acids (McCoy, Meyer, and Rose, 1935-36). Thus a practicable method is available for determining the nutritive importance of the amino acids by omitting them from the food one at a time. Such experiments are under way or have been completed in this laboratory for each of the protein components. The results will be presented in a series of papers. The present communication is concerned with the importance of glycine and serine.

With respect to glycine, considerable evidence is recorded in the literature favoring its dispensability. Wiechowski (1906), Ringer (1911-12), and others have reported that rabbits and goats, when fed relatively large quantities of benzoic acid, excrete more glycine in the form of hippuric acid than can be derived from the proteins catabolized. The origin of the excess glycine is unknown; but McCollum and Hoagland (1913-14), Lewis (1914), and Shiple and Sherwin (1922) have shown that a considerable portion of the nitrogen which under ordinary circumstances appears in the urine as urea, may, after relatively large doses of benzoic acid, be diverted from its usual path, and utilized in the formation of hippuric acid. These observations have led to the general impression that glycine may be synthesized from ammonia and non-nitrogenous materials, or from other amino acids. Furthermore, the fact has sometimes been emphasized that casein, which is relatively

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low in glycine, serves admirably for purposes of growth, both in man and in animals. In like manner, gliadin and zein, which are supposed to be devoid of glycine, are made satisfactory for growth by suitable supplementation without the addition of glycine.

On the other hand, the evidence is not all in favor of the dietary dispensability of this amino acid. Griffith and Lewis (1923) have demonstrated that the amount of preformed glycine present in the diet is a determining factor in the *rate* of hippuric acid excretion (and probably its synthesis) in rabbits. More recently, Griffith (1929, 1929-30) has shown that the growth of young rats may be inhibited by the inclusion of sufficient benzoate in the food unless glycine as such, or in the form of protein, is supplied in amounts sufficient to detoxicate the benzoate and meet the needs of tissue synthesis. These results suggest that if the animal organism is able to synthesize glycine at all, the quantity which may be so produced is quite limited.

No information is available concerning the rôle of serine in growth.

#### EXPERIMENTAL

The composition of the amino acid mixture (Mixture XII-a) employed in this investigation is shown in Table I. It is identical with Mixture XII (*cf.* McCoy, Meyer, and Rose (1935-36)) except that serine and glycine were omitted. The make-up of the diets is shown in Table II. Each diet supplied 18 per cent of "effective" amino acids including glucosamine. The vitamin B factors were furnished to each animal in the form of two pills daily, each containing 75 mg. of milk concentrate and 50 mg. of tikitiki extract. The daily intake of nitrogen from the vitamin pills amounted to approximately 4 mg., and was the only nitrogen of unknown kind in the rations. It will be observed that Diets 25 to 28 inclusive carried 0.5 per cent of threonine, while Diets 29 to 32 inclusive contained 0.6 per cent of this amino acid. The higher level is probably nearer the minimum amount necessary to induce maximum growth.

The results of the experiments are shown in Charts I and II. In Table III are recorded the total change in body weight and the total food consumption of each animal. *An examination of the growth curves demonstrates that the omission of either glycine or*

*serine, or of both of these amino acids, exerts no inhibitory effect upon growth.* The second series of experiments (Chart II) happened to have been conducted at a time when the weather was extremely

TABLE I  
*Composition of Amino Acid Mixture*

|                           | Mixture XII-a      |         |
|---------------------------|--------------------|---------|
|                           | Active amino acids | As used |
|                           | gm.                | gm.     |
| Glycine.....              | 0                  | 0       |
| Alanine.....              | 1.90               | 3.80*   |
| Valine.....               | 8.00               | 16.00*  |
| Leucine.....              | 9.00               | 9.00    |
| Isoleucine.....           | 4.00               | 8.00*   |
| Norleucine.....           | 1.25               | 2.50*   |
| Proline.....              | 8.00               | 8.00    |
| Hydroxyproline.....       | 2.00               | 2.00    |
| Phenylalanine.....        | 3.90               | 7.80*   |
| Glutamic acid.....        | 22.00              | 22.00   |
| Hydroxyglutamic acid..... | 0                  | 0       |
| Aspartic acid.....        | 4.10               | 4.10    |
| Serine.....               | 0                  | 0       |
| Tyrosine.....             | 6.50               | 6.50    |
| Cystine.....              | 1.25               | 1.25    |
| Histidine.....            | 2.75               |         |
| "    hydrochloride.....   |                    | 3.40    |
| Arginine.....             | 5.25               |         |
| "    hydrochloride.....   |                    | 6.35    |
| Lysine.....               | 7.70               |         |
| "    dihydrochloride..... |                    | 11.55   |
| Tryptophane.....          | 2.25               | 2.25    |
| Methionine.....           | 1.75               | 3.50*   |
| Sodium bicarbonate.....   |                    | 12.86   |
|                           | 91.60              | 130.86† |

\* Racemic acids.

† 1.429 gm. of mixture are equivalent to 1.0 gm. of "effective" amino acids.

hot. Doubtless this accounts for the fact that the growth of the animals, despite the larger intake of threonine, was not greater than in the case of the first series (Chart I).



In connection with such experiments, it should be emphasized that the dispensability of an amino acid for growth purposes does not necessarily imply that it may not be required for other physiological functions, or that its nutritive significance may not be modified by extraordinary conditions of diet. Thus, when benzoic acid is administered, the synthesis of glycine by the organism may not keep pace with the combined needs of detoxication and tissue

TABLE II  
*Composition of Diets\**

|  | Diet 25 | Diet 26 | Diet 27 | Diet 28 | Diet 29 | Diet 30 | Diet 31 | Diet 32 |
|--|---------|---------|---------|---------|---------|---------|---------|---------|
|  | gm.     | gm.     | gm.     | gm.     | gm.     | gm.     | gm.     | gm.     |
| Amino acid Mixture XII-a. ....               | 22.39   | 23.11   | 21.68   | 23.82   | 22.25   | 22.97   | 21.54   | 23.68   |
| Threonine. ....                              | 0.50    | 0.50    | 0.50    | 0.50    | 0.60    | 0.60    | 0.60    | 0.60    |
| Glucosamine hydrochloride ( <i>d</i> -)..... | 1.00    | 1.00    | 1.00    | 1.00    | 1.00    | 1.00    | 1.00    | 1.00    |
| Sodium bicarbonate. ....                     | 0.40    | 0.40    | 0.40    | 0.40    | 0.40    | 0.40    | 0.40    | 0.40    |
| Dextrin. ....                                | 22.71   | 21.99   | 22.42   | 22.28   | 22.75   | 22.03   | 22.46   | 22.32   |
| Sucrose. ....                                | 15.00   | 15.00   | 15.00   | 15.00   | 15.00   | 15.00   | 15.00   | 15.00   |
| Salt mixture†. ....                          | 4.00    | 4.00    | 4.00    | 4.00    | 4.00    | 4.00    | 4.00    | 4.00    |
| Agar. ....                                   | 2.00    | 2.00    | 2.00    | 2.00    | 2.00    | 2.00    | 2.00    | 2.00    |
| Lard. ....                                   | 26.00   | 26.00   | 26.00   | 26.00   | 26.00   | 26.00   | 26.00   | 26.00   |
| Cod liver oil. ....                          | 5.00    | 5.00    | 5.00    | 5.00    | 5.00    | 5.00    | 5.00    | 5.00    |
| Glycine. ....                                | 1.00    |         | 1.00    |         | 1.00    |         | 1.00    |         |
| Serine ( <i>dl</i> -). ....                  |         | 1.00    | 1.00    |         |         | 1.00    | 1.00    |         |
|  | 100.00  | 100.00  | 100.00  | 100.00  | 100.00  | 100.00  | 100.00  | 100.00  |

\* Each diet contained 18 per cent of "effective" amino acids, including glucosamine. The vitamin B factors were supplied in the form of two pills daily, each containing 75 mg. of milk concentrate and 50 mg. of tikitiki extract. The daily intake of nitrogen from these sources amounted to approximately 4 mg.

† Osborne and Mendel (1919).

anabolism. This may account for the growth inhibition observed by Griffith (1929, 1929-30) following the inclusion of benzoate in the food. The immediate and inexorable demand for glycine imposed by the detoxication mechanism may have surpassed the upper limit of endogenous production, and thereby created a deficiency of the amino acid for growth. It is conceivable also that other factors might affect the dispensability of a protein com-

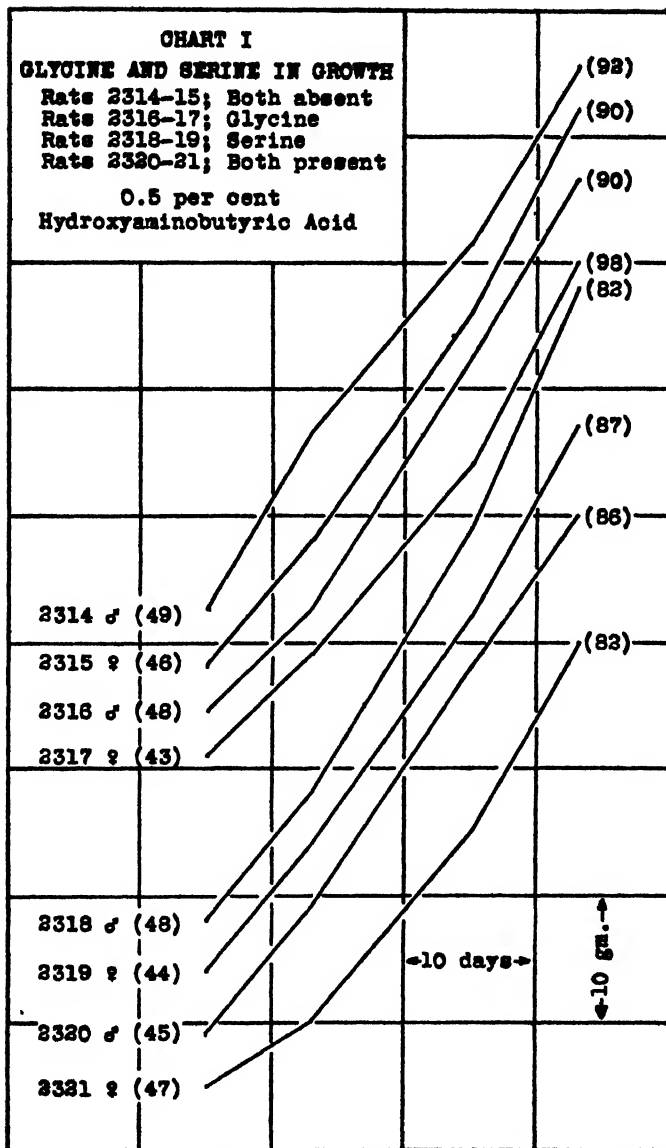


CHART I. The numbers in parentheses denote the initial and final weights of the rats.

ponent. Perhaps certain amino acids which are not required for growth may be necessary for reproduction. Furthermore, experi-

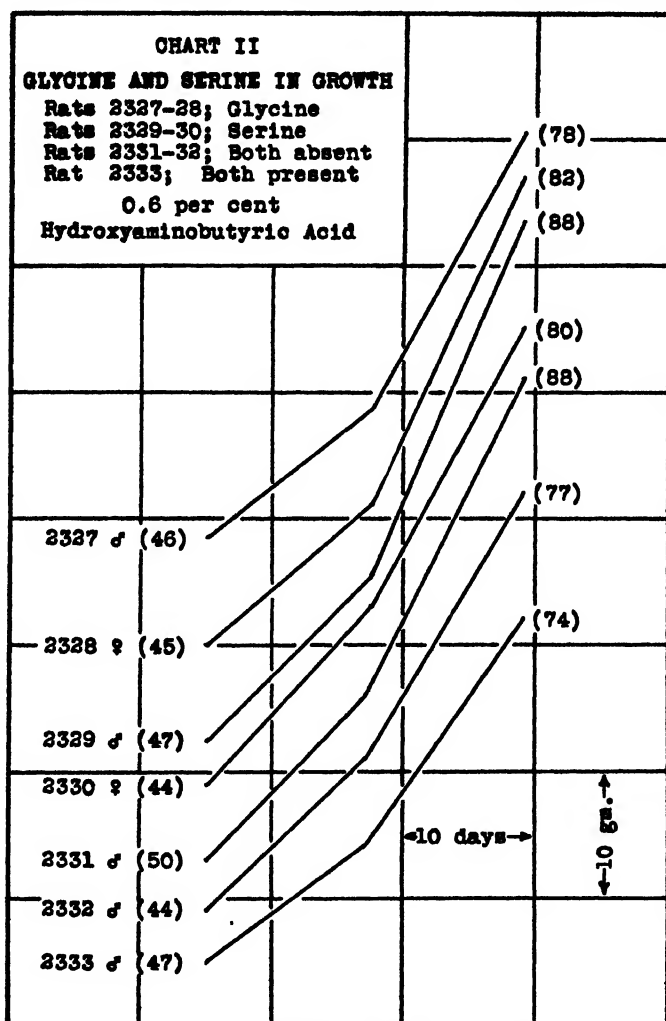


CHART II. The numbers in parentheses denote the initial and final weights of the rats.

ments carried through several generations might disclose dietary deficiencies which do not become apparent in tests of short duration.

The above considerations have not been overlooked. Hitherto we have employed the terms "indispensable" and "dispensable" in accordance with their general usage; namely, to denote whether given dietary components are or are not necessary for *growth* during periods of a few weeks or months. After the amino acids have been classified according to this criterion, we propose to formulate simpler mixtures for use in prolonged investigations involving the effects of reproduction and other factors. Possibly

TABLE III

*Total Changes in Body Weight and Total Food Intakes of Experimental Animals*

| Litter No. | Rat No. and sex | Duration of experiment | Total increase in weight | Total food intake | Supplement            |
|------------|-----------------|------------------------|--------------------------|-------------------|-----------------------|
|            |                 | <i>days</i>            | <i>gm.</i>               | <i>gm.</i>        |                       |
| 1          | 2314♂           | 28                     | 43                       | 141               | No glycine, no serine |
|            | 2315♀           | 28                     | 44                       | 122               | " " " "               |
|            | 2316♂           | 28                     | 42                       | 146*              | Glycine               |
|            | 2317♀           | 28                     | 39                       | 129               | "                     |
|            | 2318♂           | 28                     | 50                       | 131               | Serine                |
|            | 2319♀           | 28                     | 43                       | 128               | "                     |
|            | 2320♂           | 28                     | 41                       | 155*              | Glycine and serine    |
|            | 2321♀           | 28                     | 35                       | 107               | " " "                 |
|            | 2327♂           | 24                     | 32                       | 82                | Glycine               |
| 2          | 2328♀           | 24                     | 37                       | 105               | "                     |
|            | 2329♂           | 24                     | 41                       | 103               | Serine                |
|            | 2330♀           | 24                     | 36                       | 93                | "                     |
|            | 2331♂           | 24                     | 38                       | 109*              | No glycine, no serine |
|            | 2332♂           | 24                     | 33                       | 96                | " " " "               |
|            | 2333♂           | 24                     | 27                       | 79                | Glycine and serine    |

\* Some food scattered.

it may then become necessary to redefine the terms "indispensable" and "dispensable." At the present time, the distinction between an essential and a non-essential compound appears to rest solely upon the ability of the organism to accomplish its synthesis, out of the materials *ordinarily available* (*cf.* Cox and Rose (1926)), at a speed commensurate with the demands for it.

It should be borne in mind also that the same amino acids may not be needed by all species. The ultimate purpose of our animal experiments is the clarification of the human nutritive require-

ments. If the number of essential amino acids is not too large, and the amounts necessary are not excessive, it may be feasible to measure the effectiveness of suitable mixtures in the maintenance of nitrogen equilibrium in man.

#### SUMMARY

Satisfactory growth has been obtained upon diets devoid of both glycine and serine. Rats deprived of these amino acids increase in weight just as rapidly as do controls receiving similar rations supplemented with glycine and serine. The results demonstrate that the amino acids in question are *dispensable for growth purposes*.

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## THE UTILIZATION OF L-CARNOSINE BY ANIMALS ON A HISTIDINE-DEFICIENT DIET

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By taking advantage of the fact that the carnosine molecule contains histidine which is one of the amino acids known to be essential in the diet for growth, a method was afforded for studying certain aspects of the catabolism of carnosine. Such an approach would enable us to ascertain whether the histidine present in the molecule is available to the body for the various functions involved during the course of growth or whether the pathway of the catabolism of this blood pressure-lowering dipeptide of muscle tissue,  $\beta$ -alanyl-L-histidine, must follow some course in which histidine cannot be liberated.

That the catabolic fate of the histidine in this dipeptide might be different from that of the free amino acid itself appeared to be a definite possibility, since carnosine is a dipeptide in which the peptide linkage is  $\beta$  to the free amino group, in contrast to the ordinary situation that exists in peptides of  $\alpha$ -amino acids. The reports in the literature, in fact, might lead one to conclude that carnosine was not hydrolyzed into its constituent amino acids either in the intestinal tract or by the tissues. Baumann and Ingvaldsen (1), for example, reported that blood-free liver and muscle failed to hydrolyze carnosine, while Abderhalden and Geidel (2) found that an extract of a pancreas preparation had no effect on carnosine. The latter workers furthermore claimed that this  $\beta$ -amino peptide was attacked neither by trypsin-kinase nor by erepsin. On the other hand, Dietrich (cited by Parschin (3)) reported that carnosine was hydrolyzed by erepsin. Hefter (4) reported that *Bacillus pyocyaneus* attacks both carnosine and histidine with the formation of ammonia, acetic acid, and other such

split-products, but the very significant fact was noted that although this organism formed histamine from histidine none was formed from carnosine. It would thus appear that the path of the breakdown of the latter was such that free histidine was not formed. Along somewhat similar lines is the report from Parschin (3) that yeast which ordinarily forms the alcohol with 1 less carbon atom from an amino acid, and molds which usually form the hydroxy acid corresponding to the amino acid, failed to give rise to these characteristic products when allowed to act on carnosine. Only carbon dioxide and ammonia were formed. Again it would appear that the organism did not hydrolyze carnosine before metabolizing it.

The meager data reported in the literature however cannot be regarded as conclusively showing that carnosine cannot be hydrolyzed in the tissues, for only a few tissues have been studied, and it is entirely possible that enzymes for its hydrolysis may exist in some particular tissue of the body. Furthermore, some basis for suspecting that the histidine of carnosine might be available is afforded by our recent observation that the cysteine of glutathione is available for growth purposes (5). In the latter instance the cysteine must be liberated from a peptide linkage which is  $\gamma$  to the free amino group of the tripeptide, whereas in the former instance it is a question of the liberation of histidine from a peptide linkage  $\beta$  to the free amino group of the dipeptide. Of course it is clear that the two cases are not necessarily analogous. We also felt that the data on the hydrolysis of carnosine by intestinal enzymes are too limited to permit of a final conclusion.

Because of the possibility that the compound might be hydrolyzed in the intestinal tract it was deemed advisable not only to find out whether carnosine when fed could support growth of animals on a histidine-deficient diet but also whether carnosine when injected subcutaneously could serve likewise. To see whether the latter approach would be feasible, preliminary experiments were run to find out if injected histidine could support growth of animals on a histidine-deficient diet. It was found that the animals receiving histidine subcutaneously grew as well as those receiving the compound orally.

The growth with injected histidine is of additional interest at

the moment because of the claim made by Alcock (6) that injected tryptophane is not utilizable for growth purposes, a finding which was in direct contradiction to the data reported by du Vigneaud, Sealock, and Van Etten (7). Since Alcock's report this latter group has repeated its earlier experiments (8) and has again found unquestionable utilization of injected tryptophane for growth purposes. The utilization of the injected histidine in the present experiments lends support, therefore, to the contention that injected amino acids can be utilized for tissue synthesis and other functions involved during growth. It might also be mentioned that in other work in this laboratory growth of animals on a cystine-deficient diet has been obtained with diacetyl-L-cystine administered parenterally (9).

When growth with injected histidine had been established, the experiments with carnosine administered orally and subcutaneously were undertaken. In these experiments a method of preparing a histidine-free casein hydrolysate for supplying the amino acids for the diet was worked out, involving the separation of the histidine from the rest of the hydrolysate by electrodialysis. The method was based on the work of Foster and Schmidt (10) and Cox, King, and Berg (11) who employed electrodialysis for the separation and isolation of the hexone bases from protein hydrolysates. The procedure yielded a practically histidine-free casein hydrolysate and in our hands proved to be simpler and less expensive than the method which has generally been used. The latter method, which we employed in a few experiments, not reported here, is based on the precipitation of arginine and histidine by silver oxide and barium hydroxide. Furthermore, we have been able to obtain consistently by the electrodialysis method an amino acid mixture which, when cystine and tryptophane were added to it, did not support growth but which upon the addition of histidine supported growth excellently. With the silver method we have occasionally obtained batches which did not support growth properly when the histidine, cystine, and tryptophane were added. This was true even when twice the amount of histidine ordinarily used in such work was added.

As shown in Charts I and II carnosine was capable of supporting the growth of animals on the histidine-deficient diet. Both



the carnosine that was fed and that which was injected were effective. These results point to the fact that there is present in the tissues an enzyme or enzymes which can hydrolyze this  $\beta$ -amino

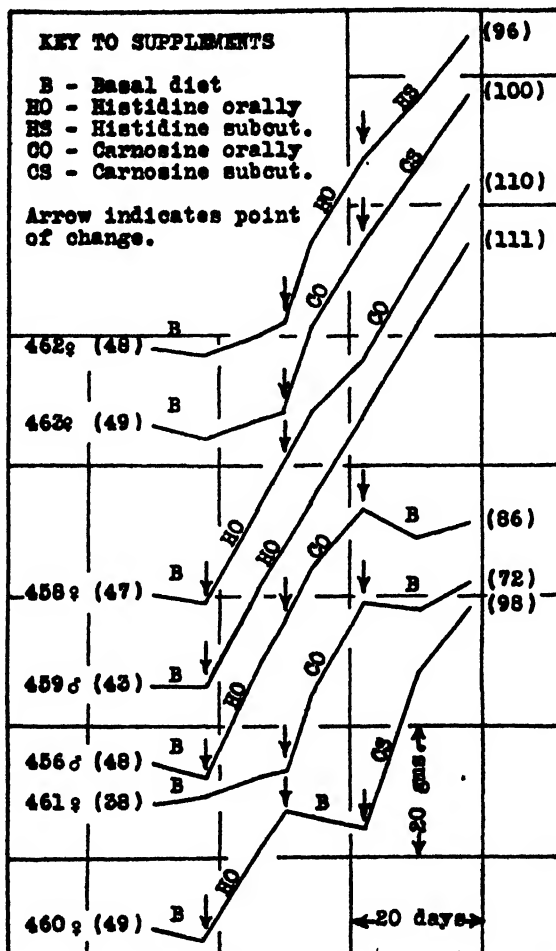


CHART I. Number and sex of rat are shown on the extreme left. The numbers in parentheses denote the initial and final weights.

peptide liberating histidine. An alternative conclusion which might conceivably be drawn is that histidine must go through the carnosine stage in order for it to be utilized. At our present

state of knowledge this would appear to be a very unwarranted assumption although not necessarily an impossibility. We should like to emphasize that the results do not rule out the possibility

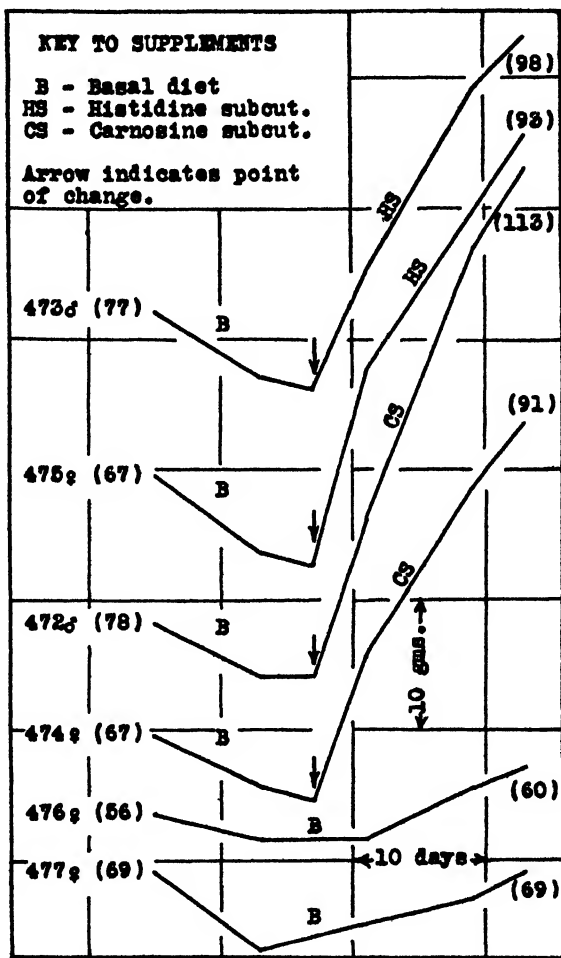


CHART II. Number and sex of rat are shown on the extreme left. The numbers in parentheses denote the initial and final weights.

of some catabolic fate of carnosine in addition to that involving a preliminary hydrolysis to its constituent amino acids, particularly when an abundance of histidine is available to the tissues.

## EXPERIMENTAL

*Preparation of Histidine-Deficient Amino Acid Mixture from Casein by Electrodialysis*—1 kilo of casein was hydrolyzed by refluxing it for 18 hours with a mixture of 1250 cc. of concentrated  $H_2SO_4$  and 3750 cc. of distilled water. The hydrolysate was diluted to about 15 liters and most of the sulfate removed by the addition of 7 kilos of ground  $Ba(OH)_2$ . The precipitated  $BaSO_4$  was washed thoroughly with three successive 6 liter portions of boiling water. The hydrolysate and wash water were combined and were concentrated *in vacuo* to about 5 liters.

The concentrate was placed in the center cell of a three cell electrodialysis set-up similar to that described by Cox, King, and Berg (11). The electrodialysis was carried out at 110 volts with sufficient resistance in the circuit to allow the passage of about 0.6 to 0.8 ampere of current. Two such set-ups were found convenient for electrodialyzing the concentrate from 1 kilo of casein.

Current was allowed to flow with frequent replacement of the cathode solution by distilled water until no histidine remained in the center cell. The Kapeller-Adler modification of the Knoop test for histidine (12) was used. Since a good test was difficult to obtain in the concentrated center cell solution, a better indication of the complete removal of the histidine was afforded by frequent tests made on the cathode solution. To effect the removal of the basic amino acids in a minimum of time the cathode was kept acid in reaction. For this purpose Foster and Schmidt (10) bubbled carbon dioxide through the cathode solution during the electrodialysis. Since in our preparation sulfate had to be removed eventually, addition of a few drops of sulfuric acid from time to time to the cathode solution served as a more convenient method of keeping the cathode solution at a pH of about 5 or 6. If the cathode is allowed to become alkaline, histidine ionizes as a negatively charged ion and moves back into the center cell as would be expected from the amino acid dissociation curves of Foster and Schmidt. Similar precautions must be taken in controlling the pH of the cathode in the steps to follow.

When the center cell was free of histidine, the cathode solutions containing the arginine, lysine, and histidine, which were removed during the electrodialysis, were combined as Fraction I and the center and anode cells, which were free of histidine, were removed and were combined as Fraction II.

Fraction I, which was slightly alkaline to phenolphthalein, was concentrated to a sufficiently small volume and was electro-dialyzed from the center cell. The electrodialysis was continued with frequent replacement of the cathode solution by distilled water until the first faint test for histidine was obtained at the cathode. The current was stopped and the cathode solutions were combined with Fraction II. These cathode solutions contained practically all of the arginine and lysine but only traces of the histidine. At the initial alkaline reaction of the center cell arginine and lysine migrated readily to the cathode but the histidine, being ionized as a negative ion at this pH, was unable to move to the negative pole. As the basic arginine and lysine were removed from the center cell, however, the pH of this solution dropped sufficiently to allow the histidine to migrate as a positive ion.

Electrodialysis of the acid solution remaining in the center cell was continued with fresh distilled water in the cathode cell until all of the histidine was removed. The histidine-free material remaining in the center and anode cells was combined with Fraction II.

The sulfate was removed quantitatively from Fraction II with hot saturated  $\text{Ba}(\text{OH})_2$  solution. The clear filtrate was concentrated *in vacuo*. As the amino acids separated during concentration they were filtered off and dried, the more soluble amino acids being finally left as a slightly viscous syrup. This was further dried before a fan and finally desiccated with acetone. All of the dry, histidine-free fractions were combined and homogenized in the ball mill. The yield from 1 kilo of casein was about 600 gm. of practically histidine-free amino acid mixture.

*Growth Experiments*—The carnosine used was synthesized by the method of Sifferd and du Vigneaud (13) and possessed the physical constants reported by them. The histidine-deficient basal diet was composed of electrodialyzed casein 8.6, *L*-cystine 0.2, *L*-tryptophane 0.2, dextrin 46.0, sucrose 15.0, lard 19.0, cod liver oil 5.0, salt mixture (Osborne and Mendel (14)) 4.0, and agar 2.0 per cent. This diet and water were furnished *ad libitum* to white rats and a record was kept of food consumption.

For oral administration the carnosine and histidine were incorporated in the vitamin pills consisting of 200 mg. of Yeast Foam Tablet Powder (Northwestern Yeast Company) and suffi-

cient water to give a dough suitable for rolling pills. For the histidine supplement each pill contained 30.94 mg. of histidine monohydrochloride (equivalent to 25 mg. of free histidine), while for the carnosine supplement each pill contained 36.5 mg. of carnosine (equivalent to 25 mg. of free histidine). The rats were

TABLE I  
*Food Consumption*

| Rat No.<br>and sex | Days  | Daily food<br>consumption | Supplement  | Rat No.<br>and sex | Days  | Daily food<br>consumption | Supplement                                |
|--------------------|-------|---------------------------|---|--------------------|-------|---------------------------|---|
|                    |       | gm.                       |   |                    |       | gm.                       |   |
| 456 ♂              | 1-8   | 3.0                       | Histidine orally<br>Carnosine "                   | 462 ♀              | 1-20  | 3.1                       | Histidine orally<br>" subcu-<br>taneously |
|                    | 9-20  | 5.3                       |   |                    | 21-32 | 6.7                       |   |
|                    | 21-32 | 7.0                       |   |                    | 33-48 | 6.8                       |   |
|                    | 33-48 | 3.8                       |   |                    |       |                           |   |
| 458 ♀              | 1-8   | 2.1                       | Histidine orally<br>Carnosine "                   | 463 ♀              | 1-20  | 3.3                       | Carnosine orally<br>" subcu-<br>taneously |
|                    | 9-20  | 5.0                       |   |                    | 21-32 | 6.5                       |   |
|                    | 21-48 | 7.3                       |   |                    | 33-48 | 8.0                       |   |
| 459 ♂              | 1-8   | 2.0                       | Histidine orally                                  | 472 ♂              | 1-12  | 4.1                       | Carnosine subcu-<br>taneously             |
|                    | 9-48  | 6.5                       |   |                    | 13-28 | 8.5                       |   |
| 460 ♀              | 1-8   | 2.5                       | Histidine orally<br>Carnosine subcu-<br>taneously | 473 ♂              | 1-12  | 4.6                       | Histidine subcu-<br>taneously             |
|                    | 9-20  | 5.4                       |   |                    | 13-28 | 7.4                       |   |
|                    | 21-32 | 3.5                       |   | 474 ♀              | 1-12  | 4.4                       | Carnosine subcu-<br>taneously             |
|                    | 33-48 | 6.6                       |   |                    | 13-28 | 7.1                       |   |
| 461 ♀              | 1-20  | 2.9                       | Carnosine orally                                  | 475 ♀              | 1-12  | 3.4                       | Histidine subcu-<br>taneously             |
|                    | 21-32 | 6.0                       |   |                    | 13-28 | 7.0                       |   |
|                    | 33-48 | 4.1                       |   |                    |       |                           |   |
|                    |       |                           |   | 476 ♀              | 1-28  | 4.0                       |   |
|                    |       |                           |   | 477 ♀              | 1-28  | 3.7                       |   |

given two pills daily at 9 a.m. and 7 p.m. so that each animal received the equivalent of 50 mg. of free histidine daily.

For parenteral administration the histidine and carnosine were injected under the skin of the back of the animal. For the histidine supplement a solution containing 41.26 mg. of histidine monohydrochloride per cc. was used and 0.5 cc. was injected at

9 a.m., 3 p.m., and 10 p.m. so that each animal received the equivalent of 50 mg. of free histidine daily. For the carnosine supplement a solution containing 48.6 mg. of carnosine per cc. was used and three injections of 0.5 cc. were made at the times given above so that the equivalent of 50 mg. of free histidine was administered daily.

Animals on the basal diet and those receiving histidine and carnosine subcutaneously received two yeast pills daily at 9 a.m. and 7 p.m., containing no amino acid supplement.

The growth results are shown in Charts I and II and the food consumption is shown in Table I. The curves in Chart I represent the results with one litter of rats, while those in Chart II represent the results with another litter.

#### SUMMARY

It has been demonstrated that carnosine administered either parenterally or orally can support the growth of the white rat on a histidine-deficient diet. The results have been taken to indicate that this  $\beta$ -amino peptide,  $\beta$ -alanyl-L-histidine, can be hydrolyzed in the tissues and that the catabolic fate of the histidine moiety of the carnosine follows the same pathway as free histidine under these conditions.

It has also been shown that injected histidine can be utilized for growth purposes.

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## THE ORGANIC ACIDS OF RHUBARB (*RHEUM HYBRIDUM*)\*

### I. ON THE MALIC ACID OF RHUBARB, WITH A NOTE ON THE MALIC ACID OF TOBACCO LEAVES

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The extensive literature on the occurrence of malic acid in plant tissues was reviewed by Franzen and Keyssner (1) in 1923. Although they found that the conclusive tests of isolation and chemical identification had been applied in relatively few cases, there is no question that this acid is widely distributed in nature and is of great importance in plant metabolism. In those instances in which observations of the optical activity have been made, the levorotatory isomer has invariably been found. Previous to 1924 indeed, when Dakin (2) discovered a simple method to resolve commercial synthetic *dl*-malic acid, *d*-malic acid was an exceedingly rare substance.

In two papers published in 1927 and in 1929, Ruhland and Wetzel (3) stated that the rhubarb plant contains not only *l*-malic acid, but the *dl* and *d* isomers as well, the *l* isomer being found chiefly in the aerial parts, the inactive isomer chiefly in the rhizome and newly developed leaf tissue. They believe that malic acid is involved in the general protein metabolism, being produced along with ammonia by deamination and oxidation of amino acids.

If the malic acid of plants originates from the protein amino acids, it should have the same optical configuration as the substances from which it is derived. Pasteur, in 1852 (4), showed

\* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington.

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that *l*-malic acid can be prepared from naturally occurring *l*-asparagine, and the configurational relationship of *l*-malic acid to the entire series of protein amino acids is now thoroughly established (5). The presence of both optical isomers of malic acid in one plant under normal conditions is, therefore, a matter of considerable significance; if verified, a modification of current views of the metabolic relationships of this acid would be necessary, and the observation would form an important exception to the general principle of asymmetric synthesis in nature.<sup>1</sup>

We have accordingly studied the relationship between the quantities of total malic acid and of *l*-malic acid in samples of rhubarb of the variety Victoria. In all cases, and in all parts of the plant, a close agreement between these quantities has been observed. There seems little doubt, therefore, that the inactive acid reported by Ruhland and Wetzel must have been something other than *dl*-malic acid, unless the metabolism of the variety of rhubarb they studied is entirely different from that examined in this laboratory.

#### EXPERIMENTAL

*Samples*—Samples of leaf and petiole tissue were collected on the dates recorded in Table I from rhubarb plants growing in the garden at this Station. The first sample consisted mostly of folded leaf blade tissue from buds only a few cm. long on which the ocrea had not broken; 50 buds were collected, freed from ocrea tissue, and promptly dried in a current of air at 80° in an enclosed oven equipped with accurate temperature control. Each of the subsequent samples consisted of twenty leaves of the same size. They were pulled from the crown, freed from any adhering basal sheath, and then rapidly separated into petioles, large veins, and leaf blades; these parts were separately sliced very thin before being dried.

<sup>1</sup> The small quantity of *d*-asparagine isolated by Piutti (6) from mother liquors obtained in the crystallization of 20 kilos of *l*-asparagine derived from vetch seedlings has been shown by Pringsheim (7) to have arisen from racemization during the evaporation of the solutions. No other authenticated case of the isolation of both *d* and *l* isomers of any natural product from a single plant species has come to our attention; von Lippmann's (8) claim with respect to *d*-tyrosine from beet seedlings was not supported by an identification and was criticised by Fischer (9).

The time of collection of the buds was arbitrarily taken as zero, and collection Samples III and IV represent successive stages of development from the original bud. Samples VI, VII, and VIII represent leaves grown from an older and more fully developed root system at a season of longer days and warmer weather. The individual leaves of these samples were about 35 days old, counting from the time at which they began to unfold.

TABLE I  
*Total Malic and l-Malic Acid Content of Rhubarb Tissues*  
The data are expressed as per cent of the dry weight.

|           | Sample No. | Date     | Total malic acid | l-Malic acid |
|-----------|------------|----------|------------------|--------------|
| Buds      | I          | Apr. 9   | 0.70             | 0.65         |
| Blades    | III        | " 30     | 1.64             | 1.43         |
|           | IV         | May 14   | 2.64             | 2.48         |
| Veins     | VI         | June 28  | 2.68             | 2.65         |
|           | VII        | Aug. 8   | 3.50             | 3.44         |
|           | III        |          | 8.40             | 8.53         |
|           | IV         |          | 14.8             | 14.5         |
|           | VI         |          | 14.4             | 14.8         |
| Petioles  | VII        |          | 12.9             | 12.0         |
|           | III        |          | 17.0             | 17.8         |
|           | IV         |          | 25.2             | 24.3         |
|           | VI         |          | 25.0             | 22.1         |
|           | VII        |          | 18.1             | 17.8         |
| Rhizomes* | VIII       | Sept. 26 | 18.0             | 17.4         |
|           | VIII       |          | 0.28             | 0.25         |

\* The determinations on this sample represent analyses of an extract that had been extensively treated with norit to remove coloring matter which rendered the polarimetric method impossible. Considerable loss of malic acid occurred during the purification. The true malic acid content of the tissue as found by direct analysis was 0.52 per cent.

### *Methods*

The dried acidified tissue was extracted with ether according to the technique of Pucher, Vickery, and Wakeman (10) and the extracts were analyzed for total malic acid by the method of the same authors (11). This method depends on the oxidation of malic acid by means of potassium permanganate and potassium bromide to a steam-volatile, bromine-substituted substance which

forms an insoluble dinitrophenylhydrazine derivative. The reaction is highly specific; none of the common organic acids interferes when due precautions are taken. *l*-Malic acid was determined by the polarimetric method of Dunbar and Bacon (12), as modified by Vickery and Pucher (13). Determinations of the total organic acidity and of citric and oxalic acids were also made (10, 11).

### Results

The data collected in Table I show agreement within the limits of accuracy of the methods between the *l*-malic acid and the total malic acid content of the different tissues. Attention is especially directed to the data on the rhizomes and buds. These are the particular tissues in which Ruhland and Wetzel believe the malic acid is predominantly, if not exclusively, of the inactive variety. The methods of analysis employed by the Leipsic investigators

TABLE II

#### *Organic Acid Composition of Rhubarb Tissues*

The data are expressed as per cent of total organic acids.

|               | Sample No. | Oxalic acid | Malic acid | Citric acid | Unknown acids |
|---------------|------------|-------------|------------|-------------|---------------|
| Buds.....     | I          | 3.6         | 5.2        | 26.8        | 64.4          |
| Rhizomes..... | VIII       | 55.3        | 3.5        | 5.6         | 35.6          |

have not been published, and there is no record in their papers of the isolation and identification of the substance in question. The discrepancy between the present results and theirs may be accounted for, however, by a lack of specificity in the methods they employed, and by the technical difficulties presented by the analysis of these tissues. Table II shows that *l*-malic acid makes up respectively only 5.2 and 3.5 per cent of the total organic acidity, and that large proportions of acids of unknown nature are present. Thus the determinations are carried out under very unfavorable conditions and only highly specific methods could be expected to yield trustworthy results.

The possibility that misleading results may be obtained by the methods employed by the Leipsic school may be further illustrated by some observations of Schwarze (14) who has recorded the presence of a considerable proportion of *dl*-malic acid in the

leaf of the tobacco plant. A careful examination in our laboratory (13) of the malic acid obtained by the method of ester distillation from a number of 50 kilo lots of tobacco leaves does not support this contention. The main fractions of distilled malic ester, which amounted in two specific cases to 565 and 425 gm., were found to consist of *l*-diethyl malate of specific rotations  $[\alpha]_D^{24} = -10.79^\circ$  and  $-10.7^\circ$  respectively. International Critical Tables give the somewhat smaller value  $[\alpha]_D^{20} = -10.18^\circ$  on the authority of Walden. Frankland and Wharton (15) give  $-10.44^\circ$  and Vickery found  $-10.46^\circ$  for a specimen obtained from alfalfa leaves.<sup>2</sup> There is little doubt, therefore, of the purity with respect to optical rotation of the *l*-malic ester isolated from tobacco leaves. The two main fractions were equivalent respectively to 98.5 and 96.3 per cent of the malic acid found by the polarimetric method in the barium salt fractions precipitated by alcohol from aqueous extracts of the leaves. If the small additional amounts of *l*-malic ester in the other distillation fractions are taken into consideration, these proportions become 101 and 103 per cent respectively. It seems to be established, therefore, that the malic acid of the tobacco leaf is exclusively the levorotatory isomer.

#### SUMMARY

An examination of the malic acid in the rhizomes, buds, petioles, and leaf blades of the rhubarb plant at several stages in its development has shown that only the levorotatory isomer of this acid is present. This observation is not in accord with the statement of Ruhland and Wetzel that *dl*-malic acid likewise occurs in this plant.

It seems probable, therefore, that the inactive acid referred to by these investigators must have been something other than *dl*-malic acid. The inference is drawn that by the methods they employed there is no sharp distinction between *dl*-malic acid and other inactive acids, and confirmation of this view is found in an examination of the malic acid of tobacco leaves which was likewise found to be exclusively levorotatory in spite of a statement to the contrary by Schwarze who employed Ruhland and Wetzel's methods.

<sup>2</sup> Unpublished data.

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## THE ORGANIC ACIDS OF RHUBARB (*RHEUM HYBRIDUM*)\*

### II. THE ORGANIC ACID COMPOSITION OF THE LEAVES

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Although it has been known since the time of Scheele that higher plants contain considerable amounts of a variety of organic acids, surprisingly little definite information is available regarding the origin, function, or fate of these substances in the organism. Bennet-Clark (1) concludes his recent exhaustive review of the rôle of organic acids in plant metabolism with the statement:

"General pronouncements on the significance of the plant acids are at present probably of little value. . . The true significance of the acid metabolism of plants may perhaps lie close to Kostychev's later point of view that these acids are the building stones from which some of the complex plant products originate. . . The plant acids are possibly convertible into amino acids and proteins, complex products such as the alkaloids, and by reduction into the parent substances of the fats and also into carbohydrates. Thus they may form some essential links in the processes by which the energy of respiration is transferred to the varied synthetic activities of the plant. It is certainly clear that they are by no means waste products of metabolism."

The present investigation was undertaken in order to obtain preliminary information with respect to the oxalic, *l*-malic, and citric acid content of rhubarb leaves. We were interested in a number of phases of the subject. The matter of the alleged presence of *dl*-malic acid in this species has been dealt with in the

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preceding paper (2). In this paper we propose to discuss the quantities of the several organic acids in the petiole, main vein, and leaf blade tissues, the concentration gradients of the acids in the parts of the leaf, the relation between the concentration of the total acids in the tissues and the titratable acidity, and also the relationship between the concentration of the several acids and of the ammonia.

The methods of analysis employed (3) have been developed in this laboratory specifically for the purpose of conducting surveys of the organic acid composition of green plants. The determinations are made upon a solution obtained by ether extraction of a sample of the previously dried tissue moistened with sufficient dilute sulfuric acid to bring it to pH 1. Under these conditions the organic acids present are quantitatively extracted. The acids are transferred to dilute aqueous alkali, the ether is evaporated, and the solution is made to a definite volume. The individual determinations are made on aliquot parts of this solution. A detailed description of the samples of rhubarb studied is given in the preceding paper.

### *Results*

Table I shows the quantities of each constituent in gm. or milliequivalents per single leaf or leaf part, or, in the case of Sample I, per single bud. Table I, accordingly, gives a survey of the data in terms of a biological unit and permits inferences to be drawn with respect to the actual changes in quantity with age, as well as comparisons of the conditions in leaves of similar age developed from rhizomes of increasing age.

The data in Sections 1 and 2 show that the leaves of Sample IV were slightly heavier than those of Sample III, but this difference was due to greater hydration; the dry weight was actually less save in the petiole. It is clear that the essential change involves an increase in both dry weight and water in the petiole during the 14 day interval between collections. The leaves of Sample VI, collected 45 days later, had developed from buds during the 35 days previous to collection. They were materially heavier than the previous sample, and contained more dry matter in each part, and considerably more water. The last sample taken 41 days later, and which had also developed from buds in the interval,

TABLE I

*Organic Acids of Rhubarb Leaves*

The data are expressed as gm. or milli-equivalents per individual leaf or leaf part.

| Sample No.<br>Collection date<br>interval, days | I<br>Apr. 9<br>0 | III<br>Apr. 30<br>21 | IV<br>May 14<br>35 | VI<br>June 28<br>80 | VII<br>Aug. 8<br>121 |
|---|------------------|----------------------|--------------------|---------------------|----------------------|
| Section 1. Fresh weight, gm.                    |                  |                      |                    |                     |                      |
| Blade   |                  | 24.1                 | 20.3               | 30.4                | 31.1                 |
| Veins   |                  | 9.6                  | 9.7                | 13.1                | 10.4                 |
| Petiole   |                  | 40.9                 | 48.8               | 73.9                | 59.9                 |
| Whole leaf                                      | 3.11             | 74.6                 | 78.8               | 117.4               | 101.4                |
| Section 2. Dry weight, gm.                      |                  |                      |                    |                     |                      |
| Blade   |                  | 3.48                 | 2.46               | 3.53                | 4.37                 |
| Veins   |                  | 0.860                | 0.695              | 0.960               | 0.995                |
| Petiole   |                  | 2.53                 | 2.66               | 4.15                | 4.66                 |
| Whole leaf                                      | 0.488            | 6.87                 | 5.82               | 8.64                | 10.0                 |
| Section 3. Water, gm.                           |                  |                      |                    |                     |                      |
| Blade   |                  | 20.6                 | 17.9               | 26.9                | 26.7                 |
| Veins   |                  | 8.70                 | 9.05               | 12.1                | 9.4                  |
| Petiole   |                  | 38.4                 | 46.2               | 69.7                | 55.3                 |
| Whole leaf                                      | 2.62             | 67.7                 | 73.1               | 108.7               | 91.4                 |
| Section 4. Total organic acids,<br>m.-eq.       |                  |                      |                    |                     |                      |
| Blade   |                  | 7.11                 | 5.89               | 10.1                | 13.4                 |
| Veins   |                  | 2.72                 | 2.79               | 4.29                | 4.11                 |
| Petiole   |                  | 11.6                 | 14.8               | 23.3                | 22.5                 |
| Whole leaf                                      | 0.97             | 21.4                 | 23.5               | 37.7                | 40.0                 |
| Section 5. Oxalic acid, m.-eq.                  |                  |                      |                    |                     |                      |
| Blade   |                  | 1.45                 | 1.74               | 5.17                | 6.50                 |
| Veins   |                  | 0.66                 | 0.79               | 1.53                | 1.36                 |
| Petiole   |                  | 3.07                 | 3.52               | 6.30                | 6.60                 |
| Whole leaf                                      | 0.035            | 5.18                 | 6.05               | 13.0                | 14.5                 |
| Section 6. Citric acid, m.-eq.                  |                  |                      |                    |                     |                      |
| Blade   |                  | 1.32                 | 0.76               | 0.85                | 1.22                 |
| Veins   |                  | 0.23                 | 0.17               | 0.26                | 0.41                 |
| Petiole   |                  | 0.55                 | 0.56               | 1.14                | 2.30                 |
| Whole leaf                                      | 0.26             | 2.10                 | 1.49               | 2.25                | 3.93                 |
| Section 7. Malic acid, m.-eq.                   |                  |                      |                    |                     |                      |
| Blade   |                  | 0.85                 | 0.97               | 1.41                | 2.29                 |
| Veins   |                  | 1.08                 | 1.54               | 2.06                | 1.92                 |
| Petiole   |                  | 6.43                 | 10.0               | 15.4                | 12.6                 |
| Whole leaf                                      | 0.05             | 8.36                 | 12.5               | 18.9                | 16.8                 |



TABLE I—*Concluded*

| Sample No.....<br>Collection date.....<br>" interval, days..... | I<br>Apr. 9<br>0 | III<br>Apr. 30<br>21 | IV<br>May 14<br>35 | VI<br>June 30<br>80 | VII<br>Aug. 8<br>151 |
|---|------------------|----------------------|--------------------|---------------------|----------------------|
| Section 8. Total known acids,<br>m.-eq.                         |                  |                      |                    |                     |                      |
| Blade.....  |                  | 3.62                 | 3.47               | 7.43                | 10.0                 |
| Veins.....  |                  | 1.97                 | 2.50               | 3.85                | 3.69                 |
| Petiole.....  |                  | 10.0                 | 14.0               | 22.8                | 21.5                 |
| Whole leaf.....   | 0.34             | 15.6                 | 20.0               | 34.1                | 35.2                 |
| Section 9. Unknown acids, m.-eq.                                |                  |                      |                    |                     |                      |
| Blade.....  |                  | 3.49                 | 2.42               | 2.67                | 3.44                 |
| Veins.....  |                  | 0.75                 | 0.29               | 0.44                | 0.42                 |
| Petiole.....  |                  | 1.60                 | 0.80               | 0.50                | 1.00                 |
| Whole leaf.....   | 0.63             | 5.84                 | 3.51               | 3.61                | 4.86                 |
| Section 10. Ash, gm.  |                  |                      |                    |                     |                      |
| Blade.....  |                  | 0.256                | 0.194              | 0.339               | 0.396                |
| Veins.....  |                  | 0.087                | 0.079              | 0.123               | 0.110                |
| Petiole.....  |                  | 0.236                | 0.268              | 0.506               | 0.487                |
| Whole leaf.....   |                  | 0.579                | 0.541              | 0.968               | 0.993                |
| Section 11. Ratio of total organic<br>acids to ash              |                  |                      |                    |                     |                      |
| Blade.....  |                  | 27.8                 | 30.4               | 29.6                | 33.9                 |
| Veins.....  |                  | 31.3                 | 35.3               | 34.9                | 37.4                 |
| Petiole.....  |                  | 49.2                 | 55.2               | 46.0                | 46.3                 |
| Whole leaf.....   |                  | 37.0                 | 43.4               | 38.9                | 40.4                 |

showed evidence of dehydration coupled with increase in dry matter in each part. In general, therefore, young rhubarb leaves appear to pass through a stage of high hydration. Leaves that develop at a later part of the season are relatively high in dry matter and low in water content.

Section 4 of Table I shows that the total organic acidity of the leaves increases with age, and also that late developing leaves contain a larger amount of acids than earlier leaves. An interesting relationship exists between the quantities of organic acids and of ash, not only with respect to each leaf part, but also with respect to the leaf as a whole. The ratios are given in Section 11 of Table I and show that the variations in the amounts of acid are closely similar to the variations in the amounts of ash. The ratios for each leaf part are substantially constant, though the ratios are different for the different parts.

The distribution of the total acidity in the several parts of the leaf is to only a minor extent affected by age. Thus  $30 \pm 4$  per cent of the acidity is found in the blade,  $11 \pm 4$  per cent in the veins, and  $59 \pm 4$  per cent in the petioles, when calculations for the entire set of data are made. Such a constancy of distribution in the leaf, regardless of age or period of development, suggests that a mechanism exists which controls this distribution. In view of the relationship between acidity and inorganic ash already mentioned, it seems probable that this mechanism operates in the same manner on both acids and inorganic cations. Whether organic cations likewise share in the relation is not known.

The data for oxalic acid (Section 5) show an increase with age in each part of the leaf, but the most striking feature is the much higher oxalic acid content of the leaf blades that were developed in the later part of the season. These contained nearly 5 times as much oxalic acid as the youngest leaf blades, while the oldest petioles contained about twice as much as the youngest. The blades of the older leaves contained approximately the same amounts of oxalic acid as the petioles of the same leaves, but more oxalic acid was present in the petioles than in the blades of the younger leaves. This observation is apparently contrary to the findings of Angerhausen (4) who states that the oxalic acid of the leaf blade is greater than that of the petiole. The discrepancy is more apparent than real. Angerhausen based his conclusion on data expressed on a concentration basis; i.e., per cent of the fresh weight. As will appear below, our own data, when expressed in an analogous manner, give rise to a similar but misleading conclusion.

The rapid synthesis of oxalic acid in the first 21 days of development of the leaf indicates that reactions in which oxalic acid is an end-product form an important phase of the acid metabolism of very young tissue. A similar observation has been made on tobacco plant tissue (5).

The situation with respect to citric acid is somewhat different. The blades of Sample IV contained considerably less citric acid than those of Sample III, and the increase in the blades of the older leaves is of a minor nature. The main veins contained approximately the same absolute amount of citric acid, regardless of the age of the plant. The petioles of the later developed leaves,

**TABLE II**  
**Organic Acids of Rhubarb Leaves**

The data are expressed as gm. or milli-equivalents per 100 gm. of water in the whole leaf or leaf part.

| Sample No.<br>Collection date<br>interval, days | I<br>Apr. 9<br>0 | III<br>Apr. 30<br>21 | IV<br>May 14<br>35 | VI<br>June 28<br>30 | VII<br>Aug. 8<br>131 |
|---|------------------|----------------------|--------------------|---------------------|----------------------|
| <b>Section 1. Dry weight, gm.</b>               |                  |                      |                    |                     |                      |
| Blade.....                                      |                  | 16.9                 | 14.2               | 13.1                | 16.4                 |
| Veins.....                                      |                  | 7.5                  | 7.7                | 8.0                 | 10.6                 |
| Petiole.....                                    |                  | 6.6                  | 5.8                | 5.95                | 8.4                  |
| Whole leaf.....                                 | 18.7             | 10.15                | 7.96               | 7.95                | 10.97                |
| <b>Section 2. Total organic acids, m.-eq.</b>   |                  |                      |                    |                     |                      |
| Blade.....                                      |                  | 34.6                 | 33.0               | 37.4                | 48.5                 |
| Veins.....                                      |                  | 31.2                 | 30.8               | 35.6                | 43.8                 |
| Petiole.....                                    |                  | 30.2                 | 31.9               | 33.4                | 40.7                 |
| Whole leaf.....                                 | 37.0             | 31.64                | 32.1               | 34.6                | 43.9                 |
| <b>Section 3. Oxalic acid, m.-eq.</b>           |                  |                      |                    |                     |                      |
| Blade.....                                      |                  | 7.05                 | 9.7                | 19.2                | 24.4                 |
| Veins.....                                      |                  | 7.6                  | 8.7                | 12.7                | 14.5                 |
| Petiole.....                                    |                  | 7.9                  | 7.6                | 9.1                 | 11.9                 |
| Whole leaf.....                                 | 1.34             | 7.65                 | 8.28               | 11.95               | 16.0                 |
| <b>Section 4. Citric acid, m.-eq.</b>           |                  |                      |                    |                     |                      |
| Blade.....                                      |                  | 6.4                  | 4.4                | 3.2                 | 4.6                  |
| Veins.....                                      |                  | 2.6                  | 1.9                | 2.2                 | 4.4                  |
| Petiole.....                                    |                  | 1.3                  | 1.2                | 1.6                 | 4.2                  |
| Whole leaf.....                                 | 9.9              | 3.10                 | 2.04               | 2.07                | 4.30                 |
| <b>Section 5. Malic acid, m.-eq.</b>            |                  |                      |                    |                     |                      |
| Blade.....                                      |                  | 4.1                  | 5.45               | 5.25                | 8.6                  |
| Veins.....                                      |                  | 12.4                 | 17.0               | 17.1                | 20.5                 |
| Petiole.....                                    |                  | 16.7                 | 21.6               | 22.2                | 22.8                 |
| Whole leaf.....                                 | 1.9              | 12.3                 | 17.1               | 17.4                | 18.4                 |
| <b>Section 6. Total known acids, m.-eq.</b>     |                  |                      |                    |                     |                      |
| Blade.....                                      |                  | 17.55                | 19.55              | 27.65               | 37.6                 |
| Veins.....                                      |                  | 22.6                 | 27.6               | 32.0                | 39.4                 |
| Petiole.....                                    |                  | 25.9                 | 30.4               | 32.9                | 38.9                 |
| Whole leaf.....                                 | 13.14            | 23.05                | 27.4               | 31.4                | 38.7                 |
| <b>Section 7. Unknown acids, m.-eq.</b>         |                  |                      |                    |                     |                      |
| Blade.....                                      |                  | 17.05                | 13.45              | 9.75                | 10.9                 |
| Veins.....                                      |                  | 8.6                  | 3.2                | 3.6                 | 4.4                  |
| Petiole.....                                    |                  | 4.3                  | 1.5                | 0.5                 | 1.8                  |
| Whole leaf.....                                 | 23.86            | 8.59                 | 4.7                | 3.2                 | 5.2                  |
| <b>Section 8. Ash, gm.</b>                      |                  |                      |                    |                     |                      |
| Blade.....                                      |                  | 1.25                 | 1.03               | 1.26                | 1.49                 |
| Veins.....                                      |                  | 1.00                 | 0.87               | 1.02                | 1.17                 |
| Petiole.....                                    |                  | 0.62                 | 0.56               | 0.73                | 0.88                 |
| Whole leaf.....                                 |                  | 0.855                | 0.740              | 0.890               | 1.09                 |

however, contained about 4 times as much citric acid as those of the youngest.

Malic acid, like oxalic acid, occurs in larger amounts in all parts of the leaves of later development than in the younger leaves, approximately twice as much being present in each leaf part of Sample VII as in Sample III.

The unknown acids, that is the difference between the total organic acidity and the sum of the oxalic, citric, and malic acids, occur in greater amount in the young tissues than in the older, and a larger amount occurs in the blades than in the veins or petioles.

In general, the predominating acid of the leaf blade is oxalic; the group of unknown acids taken together makes up a quantity even greater than the oxalic in the youngest leaves, but is distinctly less important quantitatively in the leaves of later development. Malic and citric acids are present in smaller amounts.

The predominating acid of the vein tissue is malic acid with oxalic acid in second place. Citric acid and the group of unknown acids occupy minor positions.

The petiole tissue likewise shows a marked preponderance of malic acid, oxalic acid again being a close second in quantity. Citric acid is in third place, and, save in the youngest tissue, the unknown group is present only in small amounts.

The acid composition of each part of the leaf undergoes continuous change as the age of the root system increases, and, in view of the constancy of the relative amounts of total organic acids in the several parts of the leaf, it is clear that there is a close interrelationship in the metabolism of the three chief acids.

The data in Table II have been calculated in terms of the concentration in gm. or milli-equivalents per 100 gm. of water in each leaf part, or in the whole leaf. The figures are therefore analogous to and are also closely similar in order of magnitude to results calculated on the basis of percentage of the fresh weight. They refer more accurately, however, to the fact that the organic acids occur for the most part in solution in the cells as acid salts<sup>1</sup>

<sup>1</sup> If it be assumed that the acids dissociate in the cell sap essentially as they do in water, then at pH 3.3, the approximate reaction of petiole sap, malic acid is present to the extent of 62 per cent as free acid, 35 per cent as acid salt, and 2 per cent as neutral salt; oxalic acid, 2 per cent as free acid, 90 per cent as acid salt, and 8 per cent as neutral salt; citric acid,

and permit comparisons of the relative concentrations of the acids in the different parts of the leaf.

Section 2 of Table II shows that organic acids occur in relatively high concentration in the bud tissue, at a somewhat lower concentration in the young leaves, but at gradually increasing concentration in the leaves of later development until ultimately the initial concentration in the bud is exceeded. Bennet-Clark and Woodruff (6) likewise observed a high concentration of acids in young tissue and a decrease with age. Although there is a slight concentration gradient diminishing from blade to vein to petiole in all the samples, the most striking feature of the data as a whole is the approximate constancy of the concentration of total acids in all parts of the leaf regardless of the age of the root system at which the leaves developed. Only the last sample shows a distinctly higher concentration than the others.

The data for the individual acids can be most clearly summarized in terms of the obvious concentration gradients present in the leaf structure. The concentration of oxalic acid, although essentially constant in all parts of the leaves of Sample III, shows a marked increase from petiole to vein to leaf blade in all the other samples. In the leaves of late development the concentration in the blade is more than twice as great as in the petiole. Citric acid shows a gradient in the same direction in the young leaves, but this becomes less apparent in the leaves of intermediate age, and disappears in the oldest leaves. In all cases, however, the concentration of citric acid is low compared to that of oxalic acid.

The unknown organic acids are also distributed in accordance with a concentration gradient which increases from petiole to blade, and in this case the gradient is exceedingly steep, there being a very much higher concentration of these acids in the

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37 per cent as free acid, 53 per cent as diacid salt, 8 per cent as monoacid salt, and 1 per cent as neutral salt. At pH 4.3, the approximate reaction of the blade tissue, malic acid is present to the extent of 13 per cent as free acid, 73 per cent as acid salt, and 13 per cent as neutral salt; oxalic acid, 60 per cent as acid salt, and 40 per cent as neutral salt; citric acid, 5 per cent as free acid, 42 per cent as diacid salt, 48 per cent as monoacid salt, and 6 per cent as neutral salt. These calculations disregard the possibility that cations may be present which precipitate a portion of one or more of the acids.

blade than in the petiole. What relationship these concentration gradients bear to possible movements of the acids within the leaf structure cannot be stated from the present evidence. Experiments such as those of Maskell and Mason (7) with the cotton plant would be required to shed light on this point.

L-Malic acid is distributed in the leaves of the rhubarb in a manner quite the opposite to that of the other acids. In all cases there is a higher concentration in the petiole than in the blade with the veins occupying an intermediate position in this respect, but nearer to the petiole than to the blade. The concentration in the blade is, in fact, of the same order as that of the citric acid, whereas the concentration in the petiole is in all cases very high, far exceeding that of the oxalic acid. It is because of the existence of this steep opposing gradient that the total acid concentration in all parts of the leaf is not far from constant.

A conclusion very different from this is reached if reliance is placed on the titratable acidity as a measure of the total acids present. Steinmann (8) and also Culpepper and Caldwell (9) have presented such data and arrive at the conclusion that the acid concentration in the petiole is materially higher than that in the leaf blade. The difference can be readily explained, however. Although the total organic acidities of the blade and petiole are not far apart, the hydrogen ion activity of the petiole is much greater than that of the blade (see Table IV) owing to the presence of a higher concentration of inorganic cations in the blade tissue (see Table II, Section 8). Moreover, malic acid occurs in high concentration in the petiole, and the reaction is such that about 60 per cent of it is present as free undissociated acid. Accordingly, direct titration of extracts of the two tissues might be expected to show an appreciable difference.

The data in Table II can be shown to illustrate this. If it be assumed that the unknown organic acids are dibasic and of a strength similar to that of malic acid, and further, that the titration end-point is at pH 8, a figure which approximates to the titratable acidity can be calculated. The data in Table III were obtained in this way, and it is clear that in all cases the calculated titratable acidity of the petiole is materially greater than that of the leaf blade, a result in complete agreement with the findings of the authors mentioned above.

*Relationship between Organic Acids and Ammonia*—The detoxication of ammonia in plants by synthesis of an amide (asparagine or glutamine) is a well known phenomenon. It has been studied by Prianschnikow (10) and by Mothes (11) in many species, and has been observed in this laboratory in the beet (12), the tobacco (13), and the tomato plant (14). An alternative method of detoxication has been suggested by Ruhland and Wetzel, who maintain that plants possessed of highly acid saps detoxify ammonia by simple neutralization with an organic acid, both oxalic and malic acids being so used. Ruhland and Wetzel (15) classify rhubarb as a typical acid plant, and state that a rapid deamination of amino acids occurs during the development of the leaf accompanied by the production of *l*-malic acid and of ammonia in

TABLE III

*Total Organic Acidity and Calculated Titratable Acidity of Rhubarb Leaves*

The data are expressed in milli-equivalents per 100 gm. of water in each leaf part.

| Sample No. | Blade                 |                     | Petiole               |                     |
|------------|-----------------------|---------------------|-----------------------|---------------------|
|            | Total organic acidity | Titrateable acidity | Total organic acidity | Titrateable acidity |
| III        | 34.6                  | 10.9                | 30.2                  | 21.2                |
| IV         | 33.0                  | 14.0                | 31.9                  | 23.2                |
| VI         | 37.4                  | 15.0                | 33.4                  | 23.2                |
| VII        | 48.5                  | 20.8                | 40.7                  | 27.8                |

approximately equivalent quantities. Under certain conditions of culture, they found that the ammonium nitrogen may increase to 50 per cent or more of the total nitrogen of the tissue with a correspondingly high production of malic and oxalic acids.

These conclusions have been seriously called in question by Bennet-Clark and Woodruff (6) on several grounds. They point out that Ruhland and Wetzel have not described the methods by which their results were obtained, and show that some of the published data are in conflict with the laws of physical chemistry. They were also able to show that certain of Ruhland and Wetzel's claims are based upon a confusion of the *concentration* of the organic acids in the tissues with the actual quantity present in an individual leaf or plant. Bennet-Clark and Woodruff themselves

found that the ammonium content of the rhubarb plants they studied bore no relationship to the organic acid present.

The data in Table IV show the concentration of ammonia and of the individual organic acids in the samples of rhubarb tissue we have examined. Although an increased ammonia concentration is clearly evident in the leaves of late development, there is no quantitative relationship between the ammonia and any one of the three determined organic acids, with the exception of the citric acid in the petioles. The possibility that citric acid behaves as a detoxicating substance for ammonia in the sense of Ruhland

TABLE IV

*Concentration of Organic Acids and of Ammonia in Rhubarb Tissue*

The data are expressed in milli-equivalents per 100 gm. of water.

|         | Sample No. | Reaction pH | NH <sub>3</sub> -N | Total organic acids | Oxalic acid | Malic acid | Citric acid |
|---------|------------|-------------|--------------------|---------------------|-------------|------------|-------------|
| Buds    | I          | 5.22        | 0.67               | 37.0                | 1.34        | 1.9        | 9.9         |
| Blade   | III        | 4.73        | 0.71               | 34.6                | 7.05        | 4.1        | 6.4         |
|         | IV         | 4.27        | 0.91               | 33.0                | 9.7         | 5.5        | 4.4         |
|         | VI         | 4.21        | 2.39               | 37.4                | 19.2        | 5.3        | 3.2         |
|         | VII        | 4.10        | 3.06               | 48.5                | 24.4        | 8.6        | 4.6         |
| Petiole | III        | 3.26        | 0.62               | 30.2                | 7.9         | 16.7       | 1.3         |
|         | IV         | 3.20        | 1.23               | 31.9                | 7.6         | 21.6       | 1.2         |
|         | VI         | 3.26        | 1.82               | 33.4                | 9.1         | 22.2       | 1.6         |
|         | VII        | 3.33        | 1.64               | 40.7                | 11.9        | 22.8       | 4.2         |
| Rhizome | VIII       | 3.67        | 4.02               | 36.0                | 9.6         | 19.1       | 4.0         |
|         | VIII       | 5.56        | 2.26               | 82.5                | 45.7        | 2.9        | 4.6         |

and Wetzel is rendered remote, however, by the data for the leaf blades which show a decrease in citric acid concentration with increasing age of the plant coupled with an increase in ammonia concentration. Certainly none of our specimens contained any such quantities of ammonia as would correspond to the 50 per cent of the total nitrogen mentioned by Ruhland and Wetzel, and there is no correspondence whatever between the ammonia content of the samples of rhubarb we have studied and those described by them. Our experience in this connection confirms and extends that of Bennet-Clark and Woodruff.



## SUMMARY

The rhubarb leaf contains *l*-malic, oxalic, and citric acids together with acids of unknown nature. The composition differs in different parts of the leaf and is profoundly influenced by the age of the leaf and by the season in which it has developed.

The group of unknown acids predominates in the blades of the younger leaves, oxalic acid being present in next smaller amount. In the blades of leaves developed late in the season, oxalic acid predominates over the unknown acids. *l*-Malic and citric acids are present in small amounts in the blades.

The predominating acid of the main veins is *l*-malic with oxalic in second place. Citric acid and the unknown acids occupy minor positions.

The predominating acid of the petiole is *l*-malic with oxalic in second place. Citric and the unknown acids are present in small amounts.

The concentration data show that oxalic, citric, and the unknown acids occur in a concentration gradient that *increases* from petiole to vein to blade. *l*-Malic acid, on the other hand, is present in a concentration gradient that *decreases* from petiole to vein to blade. As a result, the concentration of the total organic acids is not far from constant in all parts of the leaf; there is only a slight gradient of the total organic acidity which increases from petiole to blade. It is shown that this conclusion is not at variance with the results of direct titration of extracts of the tissues; the titratable acidities of the blades and petioles calculated from the present data are in agreement in relative order of magnitude with those reported by Steinmann and by Culpepper and Caldwell.

Determinations of the ammonia in the tissues showed that the concentration is in all cases quite low, and that there is no correlation whatever with the concentration of any individual acid nor with the total acidity. This observation confirms and extends the results of Bennet-Clark and Woodruff and, like theirs, does not agree with the conclusions of Ruhland and Wetzell.

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# STUDIES ON THE MERCAPTURIC ACID SYNTHESIS IN ANIMALS

## II. THE RÔLE OF BILE IN THE ABSORPTION AND DETOXICATION OF BROMOBENZENE AND NAPHTHALENE IN THE DOG

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Wieland and Sorge (1) have shown that naphthalene forms a coordination complex with desoxycholic acid. This complex, naphthalenecholeic acid, was shown to be easily soluble and comparatively stable in dilute alkali. These workers have suggested that the formation of naphthalenecholeic acid *in vivo* might be the mechanism which is involved in the transportation of naphthalene across the intestinal wall. Once thus absorbed, naphthalene is then eliminated in the bile.

It has been shown, however, that naphthalene, when fed to rabbits (2, 3), dogs (4), swine (5), and rats (6), readily yields 1- $\alpha$ -naphthalenemercapturic acid in the urine. Evidence has also been presented to show that naphthalene, when fed to rabbits, dogs, and swine, yields, in the urine of these animals, ethereal sulfates and glucuronates. It is therefore clear from this that bile is not the main channel for the excretion of naphthalene in animals. If the absorption of naphthalene depends on the presence of bile in the intestinal tract, as the theory of Wieland and Sorge (1) seems to imply, total exclusion of bile from the intestine and feeding of naphthalene should result in the diminished absorption of naphthalene and, consequently, in the lowered output of 1- $\alpha$ -naphthalenemercapturic acid in the urine. It appeared to us of interest to investigate the extent of the synthesis of

1- $\alpha$ -naphthalenemercapturic acid in dogs with biliary fistulas. In view of the similarity of the metabolism of naphthalene in dogs (4) to the metabolism of bromobenzene in the same animal (7), the fate of bromobenzene in dogs with biliary fistulas was also investigated.

It has been repeatedly suggested that cystine is the precursor of the taurine of taurocholic acid (8-11). A decrease in the output of taurocholic acid in the bile on administration of rather large doses of bromobenzene to cats with biliary fistulas was interpreted to indicate the removal of cystine which normally would be converted to taurine to yield taurocholic acid (12, 13). Detailed reports of these experiments have not as yet been published. Conclusive experiments have been made to show that the extent of synthesis of bile salts in dogs is extremely sensitive to the slightest injury to the liver (14, 15). We therefore investigated the effects of bromobenzene and naphthalene on the output of taurocholic acid in the bile, and the histologic changes in the liver induced by these substances when fed to dogs with biliary fistulas.

#### EXPERIMENTAL

Adult female dogs weighing from 9 to 14 kilos were used exclusively. The dogs were fed uniform rations of canned food at 8.30 a.m.<sup>1</sup> and the urine was collected by catheterization every 24 hours before feeding. After several days of maintenance on the diet, each dog was then fed 1.0 gm. of bromobenzene or 1.0 gm. of naphthalene at 8.30 a.m. and the collection of urine was continued as before until the output of the corresponding mercapturic acids, as determined by the recently described method (16), ceased. The method for the determination of *p*-bromophenylmercapturic acid in dog urine (16) we found to be satisfactory for the determination of 1- $\alpha$ -naphthalenemercapturic acid. The results of application of the method to dog urine for 1- $\alpha$ -naphthalenemercapturic acid are shown in Table I.

<sup>1</sup> The constancy of composition of the commercial dog food used was checked by analysis of the food for nitrogen. The samples were taken at random from three different forty-eight can cartons. The results were 1.65, 1.59, 1.87 per cent nitrogen, or 10.13 per cent protein (average). The analysis supplied by the manufacturers was 10.5 per cent protein, 10.00 per cent carbohydrates, 2.5 per cent fat, and 0.5 per cent crude fiber.

After the animals were thus fed bromobenzene and naphthalene and the extent of the synthesis of mercapturic acids from the given dose was estimated, a Rous-McMaster biliary fistula (17) was established in each dog under aseptic conditions. The dogs were then fed the same food in the same amounts as before the operation. Naphthalene was fed altogether sixteen times, and bromobenzene seventeen times, to six dogs in doses of 1.0 gm., and the

TABLE I  
*Recovery of 1- $\alpha$ -Naphthalenemercapturic Acid from Water and Urine*

| Medium    | Mercap-<br>turic<br>acid<br>added | 0.01 N I <sub>2</sub> absorbed |       |                 | Hg(SC <sub>10</sub> H <sub>7</sub> ) <sub>2</sub> | Mercapturic acid<br>found by methods |                   | Per cent<br>recov-<br>ered |
|-----------|-----------------------------------|--------------------------------|-------|-----------------|---|--------------------------------------|-------------------|----------------------------|
|           |                                   | Blank                          | Final | Differ-<br>ence |   | I <sub>2</sub>                       | HgCl <sub>2</sub> |                            |
|           | mg.                               | cc.                            | cc.   | cc.             | mg.   | mg.                                  | mg.               |                            |
| Water     | 10.0                              |                                |       |                 | 8.12  |                                      | 9.10              | 91.0                       |
|           | 15.0                              |                                |       |                 | 12.50   |                                      | 14.0              | 93.3                       |
|           | 10.0                              | 0                              | 3.40  | 3.40            |   | 9.8                                  |                   | 98.0                       |
|           | 10.0                              | 0                              | 3.28  | 3.28            |   | 9.5                                  |                   | 95.0                       |
| Dog urine | 10.0                              |                                |       |                 | 8.69  |                                      | 9.74              | 97.4                       |
|           | 18.1                              |                                |       |                 | 14.72   |                                      | 16.49             | 91.1                       |
|           | 10.0                              | 0.42                           | 3.64  | 3.22            |   | 9.3                                  |                   | 93.0                       |
|           | 18.1                              | 0.49                           | 6.38  | 5.89            |   | 17.01                                |                   | 94.0                       |
| Pup urine | 16.65                             |                                |       |                 | 13.38   |                                      | 14.99             | 90.0                       |
|           | 16.65                             |                                |       |                 | 13.68   |                                      | 15.32             | 92.0                       |
|           | 16.65                             | 0.38                           | 5.77  | 5.39            |   | 15.59                                |                   | 93.6                       |
|           | 16.65                             | 0.38                           | 5.63  | 5.25            |   | 15.18                                |                   | 91.1                       |

The recoveries were made on 25 cc. of water and ZnSO<sub>4</sub>-NaOH filtrate of urine with the HgCl<sub>2</sub> procedure, and on 10 cc. of water and ZnSO<sub>4</sub>-NaOH filtrate with the I<sub>2</sub> procedure. Mercapturic acid, dissolved in 0.1 N NaOH, was added to urine before the addition of ZnSO<sub>4</sub>-NaOH. 1 cc. of 0.01 N I<sub>2</sub> is equivalent to 2.89 mg. of 1- $\alpha$ -naphthalenemercapturic acid; 1 mg. of Hg(SC<sub>10</sub>H<sub>7</sub>)<sub>2</sub> is equivalent to 1.12 mg. of 1- $\alpha$ -naphthalenemercapturic acid. The method of calculation of the final result is the same as that outlined previously (16).

corresponding mercapturic acids were isolated from the urine after each feeding. No ill effects were apparent during the feeding experiments, and the dogs appeared to be in an excellent nutritional state.

The bile was collected immediately after collection of the urine (every 24 hours) and was analyzed for alcohol-soluble sulfur (taurocholic acid) by the Schmidt-Dart method (18), concen-

TABLE II

*Metabolism of Naphthalene and Effect of Desoxycholic Acid on Extent of Synthesis of 1- $\alpha$ -Naphthalenemercapturic Acid in Dog with Biliary Fistula*

Dog S-656; July 13, 14.1 kilos; August 21, 13.9 kilos.

| Date     | Bile                    |         |              |         |                 | Urine                                    |                 |
|----------|-------------------------|---------|--------------|---------|-----------------|--|-----------------|
|          | Taurocholic acid sulfur |         | Total sulfur |         | Volume, 24 hrs. | 1- $\alpha$ -Naphthalenemercapturic acid |                 |
|          | 10 cc. bile             | 24 hrs. | 10 cc. bile  | 24 hrs. |                 | Estimated, 24 hrs.                       | Isolated, total |
| 1938     | mg.                     | mg.     | mg.          | mg.     | cc.             | mg.                                      | mg.             |
| July 15* |                         |         |              |         |                 | 401.0                                    | 181.0           |
| " 16     |                         |         |              |         |                 | 325.0                                    |                 |
| " 21†    |                         |         |              |         |                 |  |                 |
| " 22     | 2.30                    | 12.42   | 2.74         | 14.80   | 54              |  |                 |
| " 23     | 4.74                    | 47.40   | 5.10         | 51.00   | 100             |  |                 |
| " 24     | 6.04                    | 70.67   | 6.73         | 78.74   | 117             |  |                 |
| " 25     | 10.06                   | 148.89  | 10.06        | 148.89  | 148             |  |                 |
| " 26     | 12.72                   | 145.00  | 13.56        | 154.58  | 114             |  |                 |
| " 27     | 9.18                    | 115.67  | 9.80         | 123.48  | 126             |  |                 |
| " 28     | 10.22                   | 112.42  | 10.37        | 114.07  | 110             |  |                 |
| " 29‡    | 6.08                    | 51.07   | 6.69         | 56.20   | 84              | 477.7                                    | 166.0           |
| " 30     | 4.02                    | 41.00   | 4.86         | 49.57   | 102             | 374.3                                    |                 |
| " 31     | 8.21                    | 102.63  | 8.58         | 107.25  | 125             |  |                 |
| Aug. 1   | 10.16                   | 117.86  | 10.73        | 124.47  | 116             |  |                 |
| " 2      | 9.88                    | 163.02  | 10.11        | 166.80  | 165             |  |                 |
| " 3‡     | 6.32                    | 108.70  | 6.33         | 108.88  | 172             | 590.0                                    | 183.0           |
| " 4      | 6.00                    | 78.60   | 6.29         | 82.40   | 181             | 302.0                                    |                 |
| " 5      | 8.11                    | 60.83   | 8.61         | 64.50   | 75              |  |                 |
| " 6      | 9.00                    | 99.00   | 9.23         | 101.53  | 110             |  |                 |
| " 7      | 10.90                   | 132.98  | 11.32        | 138.10  | 122             |  |                 |
| " 8§     | 4.83                    | 91.77   | 5.23         | 99.37   | 190             | 408.2                                    | 110.0           |
| " 9      | 9.49                    | 123.37  | 10.07        | 139.10  | 130             | 312.0                                    |                 |
| " 10     | 9.50                    | 96.90   | 10.17        | 103.73  | 102             |  |                 |
| " 11     | 10.70                   | 96.30   | 11.23        | 101.07  | 90              |  |                 |
| " 12     | 8.20                    | 108.24  | 8.69         | 114.71  | 132             |  |                 |
| " 13     | 9.53                    | 127.70  | 9.98         | 133.73  | 134             |  |                 |
| " 14     | 10.20                   | 115.26  | 10.78        | 121.81  | 113             |  |                 |

\* 1.0 gm. of naphthalene was fed at 8.30 a.m. to a normal dog.

† Rous-McMaster biliary fistula established.

‡ 1.0 gm. of naphthalene was fed at 8.30 a.m.

§ 2.0 gm. of desoxycholic acid and 1.0 gm. of naphthalene were fed at 8.30 a.m.

|| 2.0 gm. of desoxycholic acid were fed at 8.30 a.m.

trated nitric acid and Benedict's reagent (19) being used instead of the sodium peroxide. Total sulfur was determined also, by use of nitric acid and Benedict's reagent and then according to Folin's procedure of precipitation of the sulfates with barium chloride. Total sulfur was determined as a check on the excretion of taurocholic acid and on a possible excretion of mercapturic acids in the bile after the administration of bromobenzene and

TABLE III

*Metabolism of Bromobenzene in Dog with Biliary Fistula*

Dog S-656; July 13, 14.1 kilos; August 21, 13.9 kilos.

| Date        | Bile                       |            |                |            |                    | Urine                                      |                    |
|-------------|----------------------------|------------|----------------|------------|--------------------|--|--------------------|
|             | Taurocholic acid<br>sulfur |            | Total sulfur   |            | Volume,<br>24 hrs. | <i>p</i> -Bromophenyl-<br>mercapturic acid |                    |
|             | 10 cc.<br>bile             | 24 hrs.    | 10 cc.<br>bile | 24 hrs.    |                    | Esti-<br>mated,<br>24 hrs.                 | Isolated,<br>total |
| <i>1936</i> | <i>mg.</i>                 | <i>mg.</i> | <i>mg.</i>     | <i>mg.</i> | <i>cc.</i>         | <i>mg.</i>                                 | <i>mg.</i>         |
| July 18*    |                            |            |                |            |                    | 742.0                                      | 161.0              |
| " 19        |                            |            |                |            |                    | 286.0                                      |                    |
| " 21†       |                            |            |                |            |                    |  |                    |
| Aug. 13     | 9.53                       | 127.70     | 9.98           | 133.73     | 134                |  |                    |
| " 14        | 10.20                      | 115.26     | 10.78          | 121.81     | 113                |  |                    |
| " 15‡       | 5.80                       | 90.48      | 6.02           | 93.91      | 156                | 606.3                                      | 110.0              |
| " 16        | 5.49                       | 62.58      | 5.85           | 66.69      | 114                | 286.0                                      |                    |
| " 17        | 8.90                       | 91.67      | 9.15           | 94.25      | 103                |  |                    |
| " 18        | 10.49                      | 115.89     | 10.96          | 120.56     | 110                |  |                    |
| " 19‡       | 6.00                       | 90.00      | 6.27           | 94.05      | 150                | 891.0                                      | 122.0              |
| " 20        | 5.73                       | 66.47      | 6.00           | 69.60      | 116                | 110.0                                      |                    |
| " 21        | 9.00                       | 108.90     | 9.32           | 112.77     | 121                |  |                    |

\* 1.0 gm. of bromobenzene was fed at 8.20 a.m. to a normal dog.

† Rous-McMaster biliary fistula established.

‡ 1.0 gm. of bromobenzene was fed at 8.30 a.m.

naphthalene to dogs with biliary fistulas. *p*-Bromophenylmercapturic and 1- $\alpha$ -naphthalenemercapturic acids were estimated in each 24 hour sample of urine until the analysis indicated the absence of these acids from the urine. The acids were then isolated from each sample of urine separately by the McGuinn-Sherwin method (20). The yields are reported in Tables II and III in combined form, representing the total yield from a given dose. We found that both *p*-bromophenylmercapturic and 1- $\alpha$ -



naphthalenemercapturic acids, as isolated from the urine of dogs with biliary fistulas, were much more difficult to purify than the same acids isolated by the same procedure from the same dogs before the biliary fistulas were made. After repeated analysis of the mercapturic acids obtained from the urine of dogs with biliary fistulas, the results obtained suggested the presence of impurities which could not be eliminated by the procedure of isolation of mercapturic acids from normal dog urine. We therefore subjected the mercapturic acids isolated from the urine of dogs with biliary fistulas to purification as suggested by Bourne and Young (3). At the end of the metabolic studies the dogs were fed repeated doses of 1 to 3 gm. of bromobenzene or naphthalene (a total dose of 12 to 24 gm. in a period of 2 to 3 days), were then killed under ether anesthesia, and sections of various organs were removed for histologic study.

Bromobenzene was redistilled and naphthalene resublimed before use. A portion of desoxycholic acid was isolated from ox bile, according to Wieland and Gattermann's method (21), the remainder being kindly supplied to us by Dr. L. Fieser.<sup>2</sup>

### *Comment*

For the sake of economy of space only the typical results for each experiment are presented in Tables II and III.

The data indicate that when bile is excluded from the intestine by fistula, the extent of the synthesis of either *p*-bromophenylmercapturic or 1- $\alpha$ -naphthalenemercapturic acid is not diminished. On the contrary there is a slightly greater excretion of 1- $\alpha$ -naphthalenemercapturic acid in the urine of dogs with biliary fistula compared to the amount of mercapturic acid excreted by the same dog without the fistula. When desoxycholic acid was fed together with naphthalene to dogs with biliary fistulas, the extent of the synthesis of 1- $\alpha$ -naphthalenemercapturic acid was comparable to that by the same dog without the fistula. It was observed previously (4) that, on feeding naphthalene to dogs,

<sup>2</sup> We wish to express our gratitude to Dr. L. Fieser of Harvard University for supplying the desoxycholic acid, and to Dr. H. L. Mason and Dr. H. Power of The Mayo Clinic and to the Reverend F. Power, Dr. W. H. Hamill, and Mr. Alicino of Fordham University, for the analyses of the mercapturic acids.

the feces excreted had a perceptible odor of naphthalene. Dogs with biliary fistulas, when fed the same dose of naphthalene, excreted feces without any perceptible odor of naphthalene; nor could we detect any naphthalene in the bile of dogs receiving naphthalene alone. When the same dogs received desoxycholic acid together with naphthalene, the naphthalene could be detected in the bile on acidification of the bile with hydrochloric acid and steam distillation. Desoxycholic acid, when fed alone, appeared to have no effect on the excretion of either taurocholic acid or total sulfur in the bile. In this respect, desoxycholic acid differs from cholic acid. The latter was found to increase the output of taurocholic acid in dog bile (22). It appears possible, therefore, that desoxycholic acid may participate to a certain extent in the transportation of naphthalene across the intestinal wall and its elimination in the bile, but such a participation is not the main mechanism of the absorption of naphthalene in dogs.

As will be observed from Tables II and III, feeding of naphthalene or bromobenzene to dogs with biliary fistulas produced a definite decrease in the output of total sulfur in the bile, which is entirely accounted for by the decrease in the output of taurocholic acid sulfur. It seems that the bile is not the channel for excretion of either the mercapturic acids or the ethereal sulfates derived from bromobenzene or naphthalene. The depression in the output of taurocholic acid sulfur in the bile was greater than can be accounted for by the mercapturic acid sulfur excreted in the urine. Although it is probable that the taurine of taurocholic acid is elaborated by the normal organism from cystine (22), a mere decrease in the output of taurocholic acid in the bile, on administration of naphthalene or bromobenzene, cannot be used as conclusive evidence for such a transformation.

At the completion of the investigation all the dogs were killed by bleeding under ether. The fistulas were patent in all the animals, but some obstruction had occurred in one case. Histologically, the liver of one animal was normal, whereas that of the others showed some of the changes which frequently occur following long continued drainage of bile to the outside. These changes consisted of mild passive congestion, with small areas of early focal necrosis in the portal spaces and the accumulation of bile pigment in the Kupffer cells and liver cells. The kidneys were normal.

Pending histologic study of the livers of normal dogs which were fed naphthalene and bromobenzene, the possibility is assumed that the decrease in the output of taurocholic acid by dogs with biliary fistulas may be due to hepatic injury and not to the removal of cystine as mercapturic acid.

Purified mercapturic acids were analyzed and typical results were as given in the accompanying tabulation.

|   |            | M.p.                | N    | S     | C     | H    | Mol. wt. | $[\alpha]_D^{25}$ |
|---|------------|---------------------|------|-------|-------|------|----------|-------------------|
|   |            | $^{\circ}\text{C.}$ |      |       |       |      |          | degrees           |
| 1- $\alpha$ -Naphthalene-mercapturic acid | Calculated | 170                 | 4.84 | 11.09 | 62.30 | 5.19 | 289      | -25               |
|   | Found      | 170                 | 4.73 | 11.17 | 62.35 | 5.28 | 303      | -25               |
| <i>p</i> -Bromophenyl-mercapturic acid    | Calculated | 152                 | 4.40 | 10.06 |       |      | 318      |                   |
|   | Found      | 152                 | 4.33 | 10.00 |       |      | 314      |                   |

#### SUMMARY

Doses of 1.0 gm. of bromobenzene and naphthalene were fed separately to normal dogs, and then to the same animals after biliary fistulas had been made. All dogs were maintained on the same diet throughout the experiments. The output of the corresponding mercapturic acids in the urine and of taurocholic acid and total sulfur in the bile was estimated during the experiments.

The extent of the synthesis of *p*-bromophenylmercapturic or of 1- $\alpha$ -naphthalenemercapturic acid in dogs with a biliary fistula is not diminished as compared to the extent of the synthesis of these acids in the same dogs without the fistula.

Following the feeding of bromobenzene or naphthalene to dogs with biliary fistulas, a decreased output of taurocholic acid in the bile was observed. It is suggested that such a decrease may be attributable to hepatic injury caused by the naphthalene and bromobenzene rather than to the removal of cystine for detoxication purposes.

*p*-Bromophenylmercapturic and 1- $\alpha$ -naphthalenemercapturic acids were isolated from the urine of dogs with biliary fistulas, that were fed bromobenzene and naphthalene, respectively, the acids being identified by analysis.

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## THE EFFECT OF ANTICOAGULANTS ON BLOOD LIPIDS\*

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Boyd (3) has shown that the lipid content of red blood cells separated from defibrinated blood is higher than that from oxlated blood. Other recent evidence (2, 5-8) has indicated that the lipid content of blood plasma is lowered by certain anticoagulants. From this work it has been concluded that potassium oxalate produces a decrease in the concentration of lipids in blood plasma and an increase in the percentage of lipids in the red blood cells. The usual explanation offered for this phenomenon (4) is that potassium oxalate, by increasing the osmotic pressure of plasma, draws water from the red blood cells, thus diluting plasma and concentrating the red blood cells.

The experimental data upon which this conclusion is based show that there is a considerable variation in the effect of potassium oxalate. This variation is greater than one would expect were this relatively simple explanation the correct one. The present work has shown that a number of other factors influence the effect of potassium oxalate and similar anticoagulant salts on the lipid content of the red blood cells and of plasma. Evidence was obtained that the membrane of the red blood cells is permeable both to lipids and to anticoagulant salts over a period of time and that therefore the effect of the anticoagulant salts is not as simple as it might first appear.

A study was made first of the comparative effects of heparin, hirudin, and defibrination, since it was anticipated that none of these would materially alter the lipid content of plasma and of the red blood cells. Proceeding from this, we investigated the effect of potassium oxalate in detail and made a cursory survey of

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several other anticoagulant salts. Blood used in this work was obtained from patients of the Kingston General Hospital. Extracts were prepared by the method of cold extraction evolved by Boyd (2, 3) and analyzed by the Bloor oxidative micromethods as modified by Boyd (1).

### *Red Blood Cells*

The lipid content of the red blood cells was found to be practically identical in specimens of the same blood which had been defibrinated, heparinized, or hirudinized. Defibrination was accomplished by shaking the freshly drawn blood gently with a few separated shreds of binder twine which had been previously extracted with alcohol. Too little shaking with binder twine permitted clotting and too much caused hemolysis of the red blood cells, and even after practise had been gained in this procedure most samples of plasma were slightly tinged with red. Heparinized blood was prepared by adding 1 mg. of heparin (Connaught Laboratories) to 10 cc. of blood. Hirudin proved to be a less satisfactory anticoagulant than heparin: 1 mg. per 10 cc. of blood prevented clotting for only half an hour, 2 mg. for an hour or two, and larger amounts for several days in the ice box. It appeared that the failure of hirudin to function quickly was due to its slowness in dissolving.

Forty-eight analyses were performed on the red cells of heparinized, hirudinized, and defibrinated blood. The hematocrit readings indicated that the volume of the red blood cells was lower in defibrinated than in either heparinized or hirudinized blood, it being practically identical in both of the latter. Defibrination removed some of the red cells which became enmeshed in the clot of fibrin and this no doubt accounted for the lower values for red cell volume. No significant difference could be detected in the lipid composition of the red cells separated from these three types of blood. For example, in one experiment the phospholipid values were 270 mg. per cent in defibrinated blood, 264 mg. per cent in heparinized blood, and 266 mg. per cent in hirudinized blood.

The next factor investigated was the effect of varying the concentration of heparin and hirudin upon the lipid content of the red blood cells. With portions of the same sample of blood, heparin

was added to the concentration of 1 mg. per 10 cc., 2 mg., 4 mg., and 8 mg. respectively. A second series was arranged containing respectively 1, 2, 5, and 10 mg. of hirudin per 10 cc. of blood. The lipid content of the red cells was then estimated in all samples of blood, most of which contained a greater excess of these anticoagulants than would be normally added. The volume of the red blood cells was approximately the same in all the various samples. Similarly there were no significant differences in the values of the red cell lipids: total lipid, neutral fat, total fatty acids, total cholesterol, ester cholesterol, free cholesterol, and phospholipid.

A factor which was later found of importance when considering the effect of potassium oxalate was the question of *time* during which the anticoagulant acted. Samples of heparinized blood were kept in the ice box and extracts of the red blood cells prepared from time to time, at intervals between 4 and 72 hours. In this experiment 136 analyses were performed. There was found to be a slight, though consistent, increase in the volume of the red cells after standing 24 to 48 hours and this was accompanied by a very slight decrease in the lipid content of the red cells. It would appear that heparin after 24 hours may cause a slight imbibition of water from plasma by the red cells, or that such a change occurs in heparinized blood whether or not it is due to the anticoagulant.

On the other hand the volume of the red cells of defibrinated blood tended to decrease slightly on standing, though no significant differences could be noted in the lipid values. The effect of this factor was evaluated from 96 analyses performed on different samples of blood. It would appear that defibrination injures the red cell membrane, permitting both water and lipids to diffuse out to a slight extent.

Thirty-two analyses performed on samples of hirudinized blood demonstrated that the red cells with this anticoagulant reacted similarly to the red cells in heparinized blood. It was suggested from these experiments that heparinized and hirudinized blood probably represent more closely than defibrinated blood the normal state of blood in the body, at least from the point of view of blood lipids.

*Effect of Potassium Oxalate*—It has already been shown (3) that



potassium oxalate added to defibrinated blood causes a shrinkage of the red blood cells and an increase in their lipid content. Thirty-two additional analyses on different samples of blood confirmed this earlier finding. In upwards of 200 analyses performed on different samples of blood, it was found that the immediate effect of adding potassium oxalate to heparinized blood consisted in a shrinkage in the volume of the red cells and an increase in their lipid content. In performing these experiments, one-half of the freshly drawn blood was added to a flask containing heparin and the other half to a second flask containing potassium oxalate; both samples of blood were shaken and immediately centrifuged and extracted. It would appear from these experiments that the immediate effect

TABLE I

*Effect of Varying Concentrations of Potassium Oxalate in Contact for Several Hours with Heparinized Blood on Lipid Content and Volume of Red Blood Cells*

The lipid values are expressed in mg. per cent.

| Concentration of potassium oxalate | Volume of red blood cells | Total lipid | Neutral fat | Total fatty acids | Total cholesterol | Ester cholesterol | Free cholesterol | Phospho-lipid |
|------------------------------------|---------------------------|-------------|-------------|-------------------|-------------------|-------------------|------------------|---------------|
| per cent                           | per cent                  |             |             |                   |                   |                   |                  |               |
| 0                                  | 47.7                      | 491         | 70          | 267               | 122               | 8                 | 114              | 294           |
| 0.2                                | 42.8                      | 482         | 31          | 248               | 127               | 12                | 115              | 316           |
| 0.3                                | 38.1                      | 493         | 30          | 251               | 136               | 20                | 116              | 314           |
| 0.9                                | 33.3                      | 483         | 23          | 241               | 139               | 23                | 116              | 306           |
| 1.8                                | 28.6                      | 487         | 18          | 248               | 146               | 23                | 123              | 308           |

of adding potassium oxalate consists in a shrinkage of the red cells, with an outward diffusion of water, leaving behind probably most of the lipids. Data obtained in the next experiment indicated that very shortly some of the lipids diffused out as well and the method was not sufficiently sensitive to determine whether or not some of the lipids did not immediately diffuse out with the water.

In evaluating the effect of varying concentrations of potassium oxalate, the first inkling was obtained that time was a factor of importance. Samples of heparinized blood were divided and varying amounts of potassium oxalate added to each part; the flasks were thoroughly shaken and allowed to stand several hours

before centrifuging and extracting. It was expected that with increasing amounts of potassium oxalate the volume of the red cells would become progressively less and the lipid content progressively greater. It was found that the volume did become progressively less but there was no appreciable increase in the lipid content. The results of a typical experiment are shown in Table I; over 100 analyses in all were performed. It may be calculated from the results given in Table I that potassium oxalate caused a diminution of the lipid content per red cell. This will be apparent when it is recalled that extracts of the red cells were prepared by drawing off 2 cc. and extracting this with alcohol-ether after the cells were hemolyzed. As the volume of the red cells diminished, more and more cells would necessarily be included in the aliquot removed for analysis and yet this aliquot did not contain more and more lipids. As seen in Table I, there was essentially no increase. It is obvious thus that potassium oxalate had caused an outward diffusion of lipids as well as water from the red blood cells when allowed to stand several hours in contact with the cells.

The combined results of the previous two groups of experiments indicated that *time* must be a factor of importance. This was systematically investigated by obtaining samples of heparinized blood, making an initial extract of the red cells, adding potassium oxalate to 0.3 per cent concentration, and then making further extracts after the blood had stood for varying periods in the ice box. In all, 240 analyses were performed on several samples of blood. The results of a typical experiment are depicted in Fig. 1.

It may be seen from Fig. 1 that the immediate effect of potassium oxalate was a shrinkage of the red blood cells. The maximum decrease in volume appeared to occur almost immediately. In experiments in which samples were taken at shorter intervals than those indicated in Fig. 1, the maximum decrease was found as early as 15 minutes after the addition of potassium oxalate. It may have occurred before this time, since this was the shortest interval which could be conveniently studied. During the first 8 hours the red cell volume remained at a minimum but in most experiments tended to begin to increase toward the end of this time. By 24 hours there was definite increase in volume again

and by 48 hours the initial volume had almost been reached. Coincident with the early decline in volume, there was an increase in the lipid content of the red cells per unit volume. The lipid values returned to their initial level again more quickly than the cell volume, being in most cases back to the initial by 24 hours. There was thus a roughly inverse relation between volume and

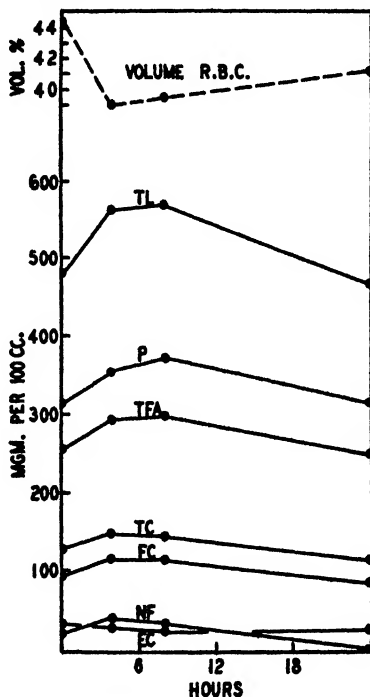


FIG. 1. The effect of potassium oxalate upon the lipid composition and volume of the red blood cells in relation to time. *TL* represents total lipid; *P*, phospholipid; *TFA*, total fatty acids; *TC*, total cholesterol; *FC*, free cholesterol; *NF*, neutral fat; *EC*, ester cholesterol.

lipid content in the earlier hours only. Since the lipid content decreased more rapidly than the volume increased, some lipids must necessarily have diffused out of the red blood cells.

It was thought possible that *changes in temperature* might have accounted for some of the effects noted above, *i.e.*, the sudden chilling of the blood by putting it in the ice box. To investigate

this, samples of heparinized blood from several patients were thoroughly mixed and divided into two portions of 100 cc. each. One portion was placed in the ice box and one left at room temperature. 1 hour later extracts were made of both specimens, potassium oxalate was added, and further extracts were made hourly to a total of ten extracts each. The results indicated that there occurred a slightly greater diminution in cell volume at room temperature and a slightly greater increase in their lipid content. Thus had the previous experiments been conducted at room temperature and bacterial and enzymatic action been prevented, the changes would have been even more marked than those registered. In other words, the changes previously observed were not due to the chilling of the red cells in the ice box.

In routine practise, blood is prevented from clotting by adding it to either *dry* or *wet* potassium oxalate; *i.e.*, 10 cc. of blood to 0.03 gm. of potassium oxalate dried carefully on the bottom of the bottle or in 0.1 cc. of distilled water. A number of experiments were conducted to determine the relative effect of potassium oxalate in these two forms. As would be expected, oxalate in solution was found to have a relatively greater effect on the volume and lipid content of the red cells than oxalate in the dry form, although the differences were very slight.

*Other Anticoagulant Salts*—The anticoagulant salts investigated included ammonium oxalate, potassium oxalate, sodium oxalate, potassium citrate, sodium citrate, potassium cyanide, sodium cyanide, potassium fluoride, and sodium fluoride. These were added in 0.3 per cent concentration to samples of heparinized blood and extracts prepared before and after addition of the anticoagulant salt. The five oxalates and citrates appeared to have very much the same effect upon red cell volume and lipid content, being analogous to that described for potassium oxalate. The cyanides produced a greater decrease in volume but not appreciably greater immediate increases in the lipid content. The fluorides produced the greatest immediate increase in cellular lipids and their effect on the cell volume lay midway between that of the oxalates and citrates and the cyanides. It would appear that the anticoagulant salts all act in a manner similar to that of potassium oxalate. The salts of lower molecular weight produced a greater effect when used in the same percentage con-

centration as the salts of higher molecular weight, which is readily explained by the greater number of available ions provided by the same concentration of the salts of lower molecular weight.

### *Blood Plasma*

In general when anticoagulants produced changes in the lipid content of plasma, these changes were the opposite to those recorded for the red blood cells. In all of the previous experiments in which the lipid content of the red cells was estimated, extracts were at the same time made of plasma.

Analyses of these extracts revealed in the first place that the lipid contents of heparinized, hirudinized, and defibrinated blood

TABLE II

*Typical Example of Effect of Potassium Oxalate (0.3 Per Cent) Upon Lipid Composition of Blood Plasma*

The results are expressed in mg. of lipid per 100 cc. of plasma.

| Time, hrs.....                      | 0    | 6    | 24   |
|-------------------------------------|------|------|------|
| Red blood cells, vol. per cent..... | 42.6 | 39.2 | 40.7 |
| Total lipid.....                    | 466  | 416  | 503  |
| Neutral fat.....                    | 199  | 165  | 191  |
| Total fatty acids.....              | 306  | 267  | 317  |
| " cholesterol.....                  | 117  | 114  | 130  |
| Ester ".....                        | 75   | 82   | 84   |
| Free ".....                         | 42   | 32   | 46   |
| Phospholipid.....                   | 100  | 82   | 112  |

plasma were practically identical. For example, in one experiment plasma phospholipid was respectively 218, 216, and 208 mg. per cent in samples of the same specimen of blood prepared with these several methods of preventing coagulation. Increasing the concentration of heparin or of hirudin did not alter the lipid values. Thus with 1, 2, 5, and 10 mg. of hirudin per 10 cc. of blood plasma phospholipid values were respectively 348, 339, 334, and 334 mg. per cent in one case. In another case with 1, 2, 4, and 8 mg. of heparin per 10 cc. of blood, the plasma ester cholesterol values were 122, 117, 114, and 124 mg. per cent respectively. No consistent detectable difference in plasma lipid values could be seen on allowing heparinized, defibrinated, or hirudinized blood to stand for periods up to 2 to 3 days in the ice box. In one experi-

ment, plasma phospholipid was 152, 156, 155, and 155 mg. per cent at 0, 4, 8, and 24 hours, respectively, in a sample of defibrinated blood.

The concentration of plasma lipids was invariably lower immediately after addition of potassium oxalate. It would be of little value to calculate the mean effect of adding potassium oxalate, since the extent of the decrease in plasma lipid percentages depended upon the concentration of the anticoagulant salt and the time over which it acted, particularly the time. Experiments designed to show the effect of potassium oxalate over a period of time from 15 minutes to 3 days showed that oxalate produced an immediate decrease in plasma lipid values which remained low for 6 to 8 hours on the average and which by 24 hours had returned to the initial level again. The results of one such experiment have been shown in Table II. The initial fall in the concentration of plasma lipids was not influenced by whether or not the blood had been stored in the ice box. A slightly greater decrease occurred when potassium oxalate was added in 30 per cent solution than when it was added in the dry form to heparinized blood.

The other anticoagulant salts, as previously listed, caused immediate decreases in the concentration of plasma lipids. The effect was relatively greater with the fluorides and the cyanides than with the oxalates and citrates.

#### SUMMARY

The effect of heparin, hirudin, defibrination, and of the ammonium, potassium, and sodium salts of oxalate, citrate, cyanide, and fluoride upon the volume of the red blood cells and upon the lipid composition of the red blood cells and of plasma was investigated by oxidative micromethods.

Lipid values were found practically the same in heparinized, hirudinized, and defibrinated blood; they were uninfluenced by the concentration of heparin or of hirudin or by allowing such blood to stand several days in the ice box.

Potassium oxalate caused an immediate decrease in cell volume, which gradually returned to the initial value on standing for 2 or 3 days. The lipid content of the red blood cells increased at first but by 1 day had returned to the initial values. The opposite

change occurred in blood plasma. Other anticoagulant salts behaved in a manner similar to that of potassium oxalate.

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# THE INFLUENCE OF INTENSE MECHANICAL VIBRATION ON THE PROTEOLYTIC ACTIVITY OF PEPSIN

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Egg albumin in aqueous solution may be denatured by sound waves of both audible (Chambers and Flosdorf (1)) and ultrasonic frequencies (Wu and Liu (2)). Chambers and Flosdorf have shown that such denaturation occurs only when the vibration is sufficiently intense to cavitate the solution. Furthermore, the reaction proceeds in the presence of specific dissolved gases, viz.  $O_2$  and  $CO_2$ ; no change occurs during sonic cavitation in atmospheres of pure  $N_2$  or  $H_2$ , or in gas-free solutions. Thus the reaction appears to be specific toward certain foreign molecules which may act either as direct absorbers of the sonic energy, or may serve as catalysts intermediary between activated water and the albumin molecules (*cf.* Fricke (3)).

The behavior of horse serum albumin, on the other hand, is quite different from that of egg albumin in that no denaturation is produced by the sonic treatment even under conditions most favorable to its denaturation by heat. It is therefore clear that whatever changes may be observed in aqueous solutions of proteins as the result of sonic cavitation, they may not be attributed to temperature increments in the liquid and cannot be accounted for in terms of ordinary thermal activation.

Since the enzyme, pepsin, has been demonstrated to have many of the properties of a protein by Northrop and his associates and others, it is a favorable material for extension of a study of the general effects of sound on relatively simple biological materials. Consequently a study has been made of the action of such vibrations on several different preparations of the enzyme with two objectives in mind: (1) the accumulation of further evidence as to



the mechanism through which sound may energize chemical transformations and (2) the application of knowledge already available relative to the action of sound on such substances as egg albumin in the hope that information bearing on the nature of the enzyme itself might be obtained.

*Enzyme Preparations*—Four types of pepsin preparations were used in the study: (1) an acid extract of hog gastric mucosa (pH 1.8); (2) a glycerol extract of pepsinogen from hog gastric mucosa. Of this preparation half was adjusted to pH 1.8 before exposure to vibration, while the other half was treated at approximately pH 7; (3) solution of Merck's u.s.p. pepsin adjusted to pH 1.8; (4) a glycerol solution (8 mg. of N per ml.) of highly purified pepsin (recrystallized three times) furnished through the courtesy of Dr. J. H. Northrop.

*Apparatus*—A magnetostriction oscillator was used to produce intense sound of 9000 cycles frequency. The vibrating element, a nickel tube, impinged directly on the solutions being studied. Both the oscillator and the method of exposure were the same as those previously described by Chambers and Gaines (4), and in their study of protein denaturation, by Chambers and Flosdorf (1).

Apparatus for treatment of solutions under increased hydrostatic pressures (Chambers (5)), under reduced gas pressures (Chambers and Flosdorf (1)), and under atmospheres other than air (Chambers and Flosdorf (1)) have all been described elsewhere.

*Method of Testing Peptic Activity*—The Mett technique as modified by Nirenstein and Schiff was used in testing peptic activity of the various preparations. While lacking the accuracy of more recent methods, this procedure was found to give reproducible results over a large number of measurements. The technique involves measurement of the distance through which digestion has proceeded along thin walled capillary tubes containing coagulated egg albumin. An empirical estimation of peptic activity follows, based on the Schütz-Borissow law that the activity of proteolytic enzyme present in a digestion mixture is proportional to the square of the number of mm. of albumin digested. All tests were carried out in quadruplicate samples and no point on an activity curve was considered significant unless checked in at least four different cases.

## EXPERIMENTAL

The effect of exposure to intense sound is fairly rapid loss of peptic activity in the case of Northrop's highly purified pepsin. The glycerol solution (50 mg. of protein per ml.) obtained from Dr. Northrop was diluted with about 250 volumes of 0.2 per cent HCl and the pH was adjusted to 1.8 before exposure. Fig. 1

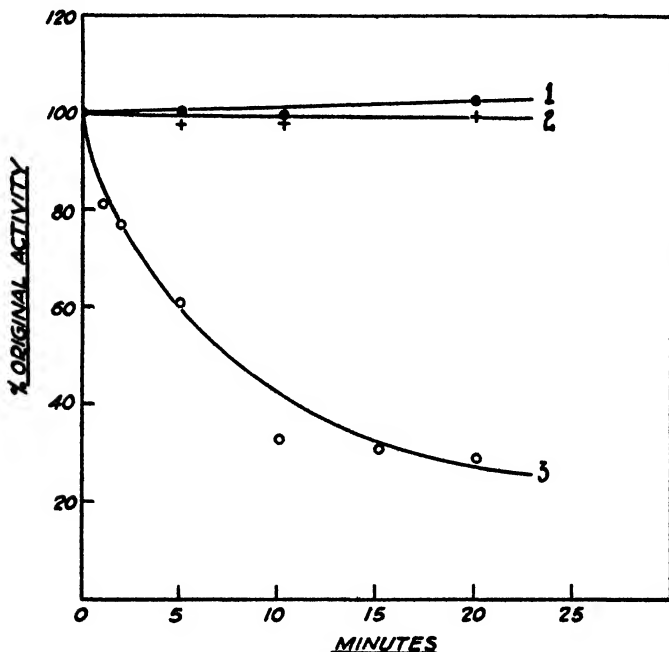


FIG. 1. The effect of sonic cavitation on the proteolytic activity of Northrop's crystalline pepsin in the presence of air (Curve 3), and of pure nitrogen (Curve 1), and in gas-free solution (Curve 2).

shows a typical activity curve obtained from such a preparation through measurements of proteolytic power at intervals during continuous vibration. Loss of peptic activity is logarithmic, following the same general relationships as those found by Williams and Gaines (6) to represent the survival of bacteria (*Escherichia coli*) during the same type of treatment. In this case the law may be expressed as

$$A = A_0 e^{-kt}$$

where  $A$  is the activity at the time  $t$ , with  $A_0$  representing the initial activity. The constant,  $k$ , is presumably determined by the acoustic energy intensity.

The results described above were obtained when the solution was vibrated in an open vessel (exposed to air). With the same methods as were used in the earlier study of albumin denaturation, portions of the pure pepsin solution were vibrated in vessels allowing (1) the complete exhaustion of dissolved gases from the solution, (2) the substitution of pure atmospheres of foreign gases such as  $N_2$  or  $H_2$ , and (3) the imposition of hydrostatic pressures up to 20 atmospheres.

When the solution was gas-free, cavitation was diminished somewhat in amount but was, nevertheless, quite vigorous. However, there was no evidence of any change whatsoever in proteolytic power of the enzyme.

Similarly, when the deaerated solution was saturated with pure  $H_2$  or pure  $N_2$ , there was no change in initial activity, although the vibration was continued for 1 hour. Thus it is evident that the inactivation reaction is dependent on the presence of a specific foreign molecule. It may not be concluded from these results that the reaction involved is an oxidation, since the situation parallels that of egg albumin denaturation in which it was found that the gas acts either as an energy acceptor or as a catalyst enabling the transfer of energy from activated water molecules to the protein. In the case of albumin at least one other molecular species may be substituted for the  $O_2$  to produce identical results.

In addition to the required presence of a specific foreign molecule, cavitation must be produced in the solution before the vibration can be utilized to inactivate the enzyme. This is demonstrated by the failure to produce a change in proteolytic power in a preparation saturated with air but vibrated under a hydrostatic pressure of 7 atmospheres. Previous studies have shown that 6 atmospheres are adequate to prevent cavitation completely.

From these results it is obvious that the conditions necessary for the sonic inactivation of highly purified pepsin in solution parallel exactly the conditions under which the sonic denaturation

of egg albumin occurs. The significance of such parallelism should not be overemphasized, but it appears to add to the already accumulated evidence that pepsin is a protein. Furthermore, it implies that sonic inactivation of the enzyme may involve the denaturation of a protein, the behavior of which toward sonic

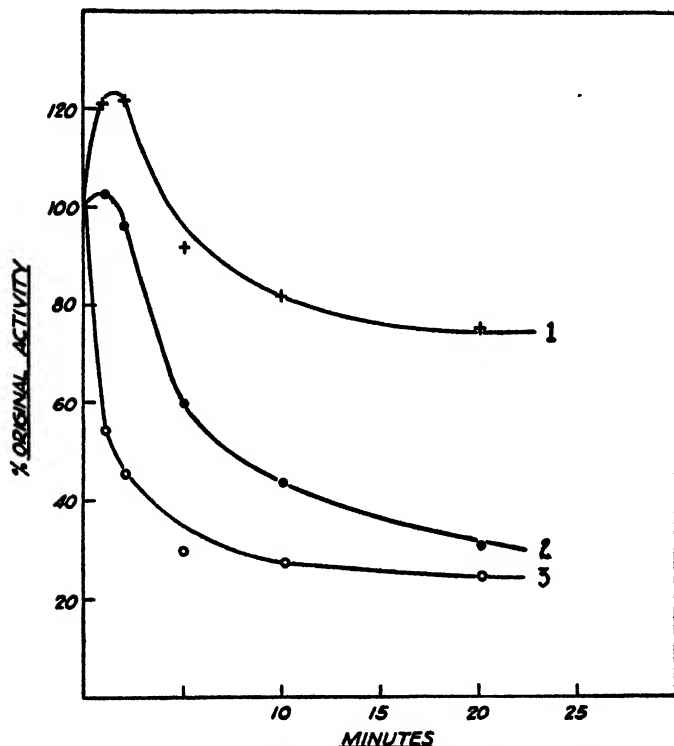


FIG. 2. The effect of sonic cavitation of the proteolytic activity of three impure pepsin preparations: Curve 1, Merck's pepsin in water; Curve 2, Merck's pepsin in acid; Curve 3, acid extract. An increased activity during the first 2 minutes of exposure is indicated in two of the three cases.

energy is identical with that of egg albumin and not at all like that of horse serum albumin.

One of the less highly purified pepsin preparations used in this study behaved toward sonic vibration in a manner identical with that of the crystallized enzyme, while all the others showed more or less striking deviations from the behavior of the pure substance.

The activity curve for a dilute HCl extract of gastric mucosa from the hog is quite similar to that determined for Northrop's preparation (Fig. 2).

On the other hand acidified solutions (pH 1.8) of Merck's u.s.p. pepsin, when exposed to sonic vibration, showed indications of an increase in proteolytic power during the first 1 or 2 minutes of vibration (Fig. 2). While the actual measured increase amounts to only about 3 per cent, there is no decrease in activity during the initial 2 minutes. Obviously this means that the normal logarithmic decrement is, during the 2 minute interval, offset by some unexpected factor operating in the opposite direction.

This trend toward increase in the initial activity of solutions of Merck's pepsin becomes still more evident if the substance be cavitated in distilled water solution. Such a preparation was exposed to vibration before acidification; the pH was adjusted to 1.8 immediately after exposure. In this case (Fig. 2) the increased activity becomes measurable during the first 3 minutes, reaching a peak at about 122 per cent of the initial activity after which the inactivation curve is approximately of the shape and position to be expected were the peak value considered the initial proteolytic power.

Still more evidence of sonic activation of the enzyme was obtained from solutions prepared by dilution of a glycerol extract of hog gastric mucosa. Four different dilutions of this material were prepared in such a manner that they contained 10, 20, 33 $\frac{1}{3}$ , and 50 per cent respectively of glycerol. Each was adjusted to pH 1.8 previous to vibration. Fig. 3 shows activity curves obtained from these four preparations. Solution A (10 per cent glycerol) shows after 2 minutes a proteolytic power about twice that of the same material before exposure to sound. On the other hand, once the activity peak is reached, the inactivation is rapid, approaching completion in 30 minutes. Solutions B (20 per cent glycerol), C (33 $\frac{1}{3}$  per cent), and D (50 per cent) show the initial rise and subsequent decrease in activity with time of exposure. However, neither the activation nor the inactivation is so marked in these cases. Furthermore, the decrease in both the initial and the final effects is directly proportional to the glycerol concentration. The ability of glycerol to inhibit de-

terioration of proteolytic activity in pepsin is already well known. One might also expect that the viscosity imparted to solutions by the presence of large quantities of the alcohol might interfere

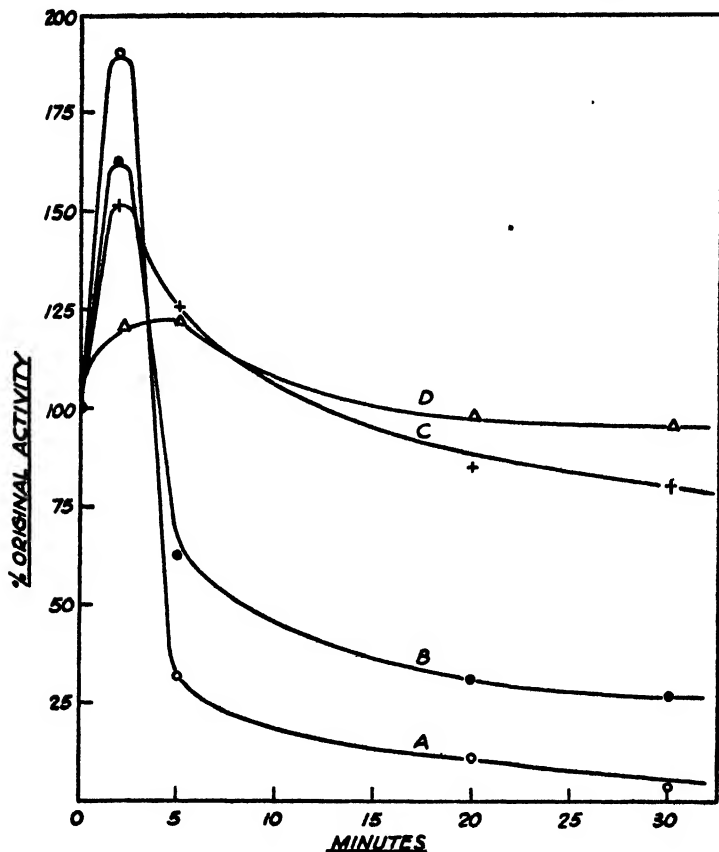


FIG. 3. The influence of sonic cavitation on the proteolytic activity of acidified (pH 1.8) glycerol extracts of gastric mucosa. Curves A, B, C, and D represent Solutions A (10 per cent glycerol), B (20 per cent), C (38½ per cent), D (50 per cent), respectively. The four curves show the inhibiting influence of high glycerol concentrations on both activation and inactivation phases.

with cavitation to a certain extent and thereby limit the effectiveness of the applied sonic energy.

While, as pointed out in an earlier portion of this paper, the

sonic inactivation observed in all the pepsin preparations may be explained as a denaturing action, no clear evidence as to the mechanism through which sound may produce the observed increases in activity has resulted from the preceding studies. In a consideration of this phenomenon it is necessary to recall that cavitation of a suspension of particles in water usually results in a further division of the suspended material and its complete dispersion. Thus oil droplets in aqueous suspension are quickly broken up into colloidal size by sonic treatment of the same kind as that imposed on the enzyme preparations. Furthermore, when suspensions containing aggregates or clusters of particles are vibrated, the first effect is a breaking up of the clumps, followed by a comminution of the dispersed individual particles. This effect of sonic cavitation is best illustrated in suspensions of such clump-forming organisms as *Staphylococcus aureus*. The groups of cells are quickly broken up, giving rise to greatly increased plate counts during the first 2 or 3 minutes, following which the normal logarithmic survival curve is observed. Survival curves for such clump-forming bacteria are thus strikingly similar to the activity curves obtained from the glycerol extracts of pepsin subjected to identical physical forces.

Therefore one may suspect that the initial increase in power to proteolyze observed in some of the impure pepsin extracts may be due to a rapid dispersal of molecular aggregates. If such groupings exist in these preparations, their dispersal might be expected to increase their availability as catalysts.

Evidence that such disruption of aggregates occurs during the first 2 or 3 minutes of cavitation has been obtained. A part of the glycerol extract preparation containing 10 per cent of glycerol was vibrated under an imposed hydrostatic pressure great enough to prevent cavitation (Fig. 4). As with the solution of Northrop's pepsin, there was no evidence of change in proteolytic activity at any time up to 1 hour of continuous treatment. This indicates that the activation process, also, is dependent upon cavitation. A second portion of the same solution was deaerated and exposed to vibration under a vacuum. In this case there was an increase to about 400 per cent of the initial activity during the first 3 minutes, after which no change occurred in either direction. Portions of the same preparation deaerated and vibrated under

atmospheres of pure  $N_2$  and  $H_2$  gave identical results except that the maximum activity was attained within 1 minute. The greater speed in these cases is probably associated with the fact

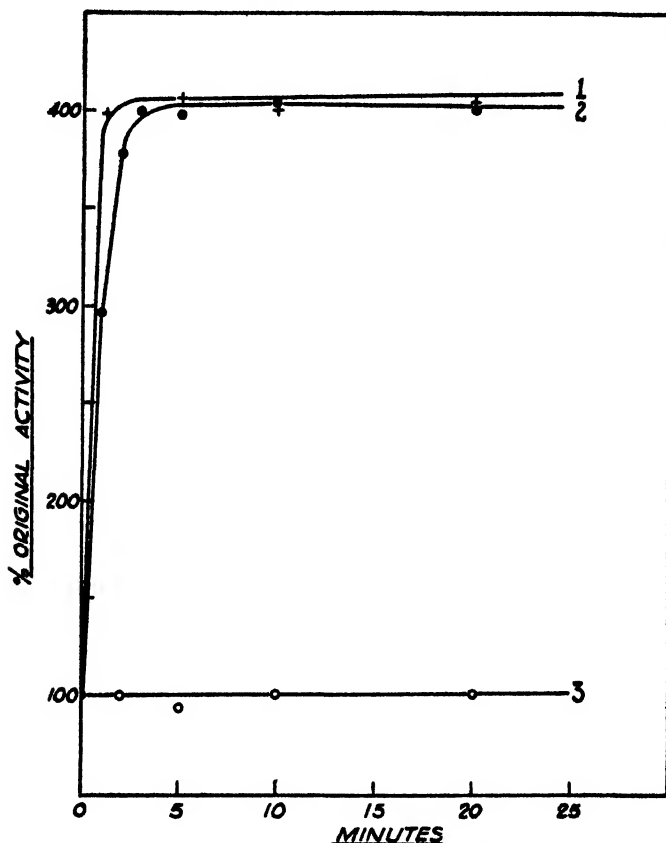


FIG. 4. Time-activity curves showing the effect obtained by sonic exposure of a 10 per cent glycerol extract in the presence of nitrogen (Curve 1) and in gas-free solution (Curve 2) (both with cavitation), and under 6 atmospheres of hydrostatic pressure with oxygen present (Curve 3). In the latter case the pressure prevented cavitation.

that cavitation is more vigorous in the presence of a gas than in deaerated solutions.

In so far, then, as sonic inactivation of pepsin is concerned, the situation with the impure glycerol preparation is the same as that



pointed out previously for the recrystallized enzyme. The phenomenon occurs only when the solution is cavitated in the presence of specific foreign gas molecules. On the other hand, the activation process occurs under all circumstances which permit cavitation and the action appears to be entirely physical, especially so in view of the fact that there is a direct relation between the speed of activation and the amount of cavitation occurring in the solution. From such evidence one may conclude tentatively that the dispersive effect of sound waves, operative through cavitation, is responsible for the observed increases in proteolytic activity of the impure enzyme. Dr. Northrop, however, has pointed out to me that destruction of an inhibitor substance, or acceleration of the rate of activation of pepsinogen through the action of sound waves, offers possible alternative explanations. The second of these two possibilities is opposed by the failure of the control solutions in the glycerol experiment (Fig. 4) to show an increase in activity with time (possibly due to an inhibiting action of the glycerol), and by the fact that the solutions were, in most cases, acidified at least 24 hours prior to the experiments, thus presumably allowing ample time for pepsinogen activation.

The conclusion that sonic activation is due to dispersion carries the implication that Northrop's pepsin and the acid extract of pepsin from hog mucosa are in a unimolecular state of distribution in the solutions studied, or at least occur in units too small for further subdivision by the sound frequencies utilized. On the other hand the preparations which showed an increased activity probably are not in a state of true solution.

#### SUMMARY

Northrop's thrice recrystallized pepsin in acid solution (pH 1.8) is inactivated by exposure to intense sound waves of 9000 cycle frequency. The relation between time of exposure and peptic activity may be expressed by the general law

$$A = A_0 e^{-kt}$$

where  $A$  represents the activity remaining at time  $t$ ,  $A_0$  being the initial activity. The inactivation reaction occurs in the presence of  $O_2$  or air, but not in pure  $N_2$  or  $H_2$ , or in deaerated

solutions. Furthermore, the reaction is inhibited by pressures sufficient to prevent cavitation even when  $O_2$  is present.

Certain, though not all, unpurified pepsin preparations show a more or less marked increase in proteolytic activity during the first 3 minutes of exposure to sound. This activation process occurs under all conditions, allowing free cavitation in the solution. In air, inactivation sets in after a maximum activity value has been reached. On the other hand the substitution of  $N_2$  or  $H_2$  (gases which do not promote protein denaturation) results in the attainment of a greater maximum activity, following which there is no further change. The increased activity is tentatively attributed to increased availability of the enzyme caused by sonic dispersal of molecular aggregates.

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## A CAPILLARY, NON-PENETRATING MICROQUINHYDRONE ELECTRODE\*

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The microquinhydrone electrode of Pierce and Montgomery<sup>1</sup> is essentially one for piercing tissues to the source of the fluid. The sharp, penetrating point is necessary under these conditions, and the use of the mercury-filled system is important in overcoming the negative capillarity of the restricted opening. Certain occasions exist when an electrode is required for use with extremely small volumes of fluid, but where the more elaborate arrangements of the Pierce and Montgomery electrode are a hindrance. Samples of not more than 3 drops, covered with a cm. of oil, have been sent to this laboratory for pH determination. From the somewhat limited need of an apparatus to be used with such minute samples has been developed a capillary quinhydrone electrode for use with fluids, or semisolid material, where the volume is small, where CO<sub>2</sub> protection is desirable, where transfer from a test-tube to a beaker is inadvisable, or where the color makes the use of indicators impossible. It may be used with less than 0.2 c.mm. of material, and permits the collection of a representative sample almost instantly. When necessary it may be sterilized at the bedside. Duplicate and triplicate determinations of known buffer solutions and of unknown, poorly buffered solutions have been found to check each other precisely. Its accuracy is comparable with that of any conventional quinhydrone system. Results may be reported within 2 or 3 minutes.

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<sup>1</sup> Pierce, J. A., and Montgomery, H., *J. Biol. Chem.*, **110**, 763 (1935).

*Method*

Platinum-iridium (85 to 15 per cent) wires of 36 gage and about 12 cm. in length were straightened by drawing them through an alcohol flame. After being rinsed in distilled water they were tipped with quinhydrone by immersing the ends in a shallow sludge of quinhydrone and acetone and allowing the solvent to evaporate.

Chemically clean quartz capillaries of about 0.33 mm. internal diameter and about 10 cm. in length were used for the collection of samples. To illustrate the general practise a description of a determination of the pH of cerebrospinal fluid will be employed. Three or four of the quartz tubes, a small flask of sterile mineral oil, and a disk of KCl-agar gel (3 per cent agar in saturated KCl solution) are taken to the bedside. As soon as the lumbar puncture has been made and the fluid flows steadily from the needle, a quartz capillary is sterilized by drawing it through an alcohol



FIG. 1. Non-penetrating microquinhydrone electrode. 1 represents the KCl-agar plug; 2, fluid for determination; 3, quinhydrone-tipped platinum-iridium wire; 4, oil; 5, quartz capillary; 6, De Khotinsky cement.

flame. It is then filled to the height of about 5 mm. by dipping one end under the oil. When the tube is withdrawn there is always a drop of oil clinging to the end, which prevents entrance of air bubbles. The oil-filled end of the capillary is then thrust with the right hand through the slip of the needle as far as it will go, while the left hand holds the gel disk. Unexhausted capillarity allows an upward flow of the cerebrospinal fluid which follows the oil. When about 1 cm. has been admitted, the tube is withdrawn quickly and instantly pushed through the gel disk. A tight plug is thus formed which excludes air and also serves as a salt bridge for electrometric determination. The other end of the column of spinal fluid is, of course, sealed by the oil. As a general thing, three samples are taken.

The tubes are then carried to the laboratory in an upright position. On arrival, the quinhydrone-tipped wire is pushed into the capillary tube through the oil to about half the length of the column of cerebrospinal fluid. The protruding end of the wire

is bent parallel to the capillary and fastened to it with a pledget of plasticin or a drop of De Khotinsky cement to prevent displacement while making electrical connections. The anterior end of the capillary, containing the KCl-agar gel, is dipped into the KCl of the calomel half-cell which previously has been heated to 38° in a water bath. Electrical connections with the potentiometer and galvanometer are made and the E.M.F. read. No significant drift in potential is ever encountered, and there is

TABLE I  
*pH of Cerebrospinal Fluid Samples*

| Patient No. | pH at 38°                    | Diagnosis                         |
|-------------|------------------------------|-----------------------------------|
| 1           | 7.46<br>7.47<br>7.43<br>7.43 | Influenzal meningitis             |
| 2           | 7.38                         | Meningococcus meningitis          |
| 3           | 7.41<br>7.40                 | " "                               |
| 4           | 7.30                         | " "                               |
| 5           | 7.34                         | " "                               |
| 6           | 7.36                         | Epilepsy                          |
| 7           | 7.27                         | Tuberculous meningitis            |
| 8           | 7.30                         | Cerebral hemorrhage               |
| 9           | 7.31                         | Acrodynia                         |
| 10          | 7.12<br>7.13                 | Pneumococcus meningitis, moribund |
| 11          | 7.48                         | Acute pyuria                      |
| 12          | 7.39                         | Meningococcus meningitis          |
| 13          | 7.20<br>7.20                 | " " moribund                      |
| 14          | 7.25<br>7.25                 | " "                               |

invariable agreement between duplicate and triplicate samples to within 0.01 to 0.02 pH. Occasionally a column of the fluid breaks and alternate layers with oil are formed. In such a case it is necessary only to push the wire below the bottom stratum of oil. Contact of oil with the quinhydrone or the platinum does not prevent electrical contact or materially increase electrical resistance, because the tangled mass of microscopic quinhydrone crystals prevents more than a film of oil on them, and this appears to break when the aqueous fluid is reached.

To test the possibility of  $\text{CO}_2$  loss in delayed determinations, triplicate samples were sometimes taken: one was tested at once, and the other two, kept in a refrigerator, were used half an hour and 1 hour apart. No significant differences were found.

The apparatus (which is shown in Fig. 1) has been tried with standard buffer solutions, and its accuracy found to be equal to that of the conventional quinhydrone electrode. A few samples of urine, saliva, and biological sera have been determined for pH in the course of ordinary routine. It has been used tentatively to determine pH of intestinal contents and excreta of rats. In these cases a small incision was made through the intestinal wall, and the tube was inserted into the mass to be sampled. The small diameter of the tube has been found useful in removing samples from punctured, rubber-stoppered serum bottles and from ampules. It is particularly valuable in taking oil-covered fluids from test-tubes.

### *Results*

In the study of the pH of cerebrospinal fluid, samples were obtained from infants in the wards of the Harriet Lane Home in the course of treatment. It was not the purpose of this work to make a study of the pH of pathological spinal fluid, and the figures in Table I are presented merely to show results obtained in a few cases. The diagnoses included with these figures do not indicate the condition of the patient when the determination was made. In two cases the patients were moribund and died shortly after the puncture was completed. In others recovery was well advanced. In still others complications existed which could have overshadowed the original diagnosis.

### SUMMARY

A capillary microquinhydrone electrode has been developed for rapid determination of the pH of small fractions of a c.mm. of biological or other fluids, and of semisolid material. When necessary it may be sterilized at the bedside. Duplicate and triplicate determinations of known buffer solutions and of unknown, poorly buffered solutions have been found to check each other precisely. The accuracy of the electrode is comparable with that of any conventional quinhydrone electrode. A series of sample determinations of pH of cerebrospinal fluid is presented.

## THE CHEMICAL ACTIVATION OF STEROLS

### II. THE CHEMICAL ACTIVATION OF CHOLESTEROL AND VARIOUS CHOLESTEROL DERIVATIVES\*

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(Received for publication, October 30, 1936)

In 1926, Bills (1) observed that cholesterol could be activated antirachitically to a slight extent by means of floridin. Later, Bills and McDonald (2) found that the antirachitic component of the resulting resinous material was a degradation product of dicholesteryl ether. This was confirmed by Kon, Daniels, and Steenbock (3). Bills and McDonald (4) also showed that this antirachitic substance was chemically different from that occurring naturally in certain fish oils or that developed by the irradiation of cholesterol, since it was not destroyed by *n*-butyl nitrite. The activating effect of floridin was reported by one of us (5) to be due to sulfur trioxide.

In a study of this activation, data were obtained which indicate, first, that cholesterol provitamin D of Waddell (6) is not the precursor of the active substance produced by the action of sulfuric acid-acetic anhydride on cholesterol or some of its derivatives; and second, that a specific relationship probably exists between chemical configuration and chemically activatable cholesterol or cholesterol derivatives.

#### *Preparation of Purified Cholesterol and Various Cholesterol Derivatives*

Purified cholesterol, m.p. 148–149°, was obtained by debrominating cholesterol dibromide prepared from recovered cholesterol which had been donated by Acetol Products, Inc., of New Bruns-

\* Journal Paper No. J385 of the Iowa Agricultural Experiment Station, Project No. 506.



wick, New Jersey. The recovered cholesterol was the relatively inactive residue which remained after the original cholesterol (Wilson, c.p.) alternately had been irradiated with ultraviolet rays and recrystallized from alcohol several times to free it from nearly all of its cholesterol provitamin D.

The cholesterol derivatives were prepared according to acceptable published procedures. All the derivatives were prepared from Wilson's c.p. cholesterol with the exception of an additional sample of cholesteryl chloride which was prepared from cholesterol purified as described above. Cholesterilene, m.p. 78–79°, and dicholesteryl ether, m.p. 195–196°, were prepared by heating cholesterol with anhydrous copper sulfate according to the directions of Mauthner and Suida (7). Cholesteryl acetate, m.p. 114–115°, was prepared by refluxing cholesterol with acetic anhydride according to the method of Anderson (8). Cholesteryl chloride was prepared by treating cholesterol with thionyl chloride which had been freshly distilled from cottonseed oil, according to the directions of Daughenbaugh and Allison (9). It was treated twice in alcohol with decolorizing carbon (norit) and recrystallized from this solvent until it melted at 95–96°.

Dihydrocholesterol was prepared by the catalytic reduction of cholesterol in alcohol according to the method of Willstätter and Mayer (10). It was purified through the acetate as follows: In a 300 cc. Erlenmeyer flask were placed 4.30 gm. of dihydrocholesteryl acetate, 1.12 cc. of concentrated sulfuric acid, and 2.4 cc. of acetic anhydride. The resulting mixture was heated for 5 hours at 85–90°, and, after cooling, the unchanged dihydrocholesteryl acetate was removed by filtration and washed with methyl alcohol. After recrystallizing from alcohol, it was saponified with alcoholic potassium hydroxide and the recovered dihydrocholesterol was recrystallized from alcohol until it melted at 141–142°.

Cholestene was prepared by reducing pure cholesteryl chloride with sodium and amyl alcohol according to the directions of Mauthner and Suida (11). As the product contained chlorine, the reduction was repeated and the resulting cholestene was recrystallized from ether-alcohol until it melted at 90–91°. Cholestenone, m.p. 79–80°, was prepared by the oxidation of cholesterol dibromide followed by debromination according to the method of Schoenheimer (12). Butyl cholesteryl ether, m.p.

78–79°, was prepared according to the directions of Bills and McDonald (2).

### *Irradiation*

The irradiation of cholesterol and the cholesterol derivatives used in this study was carried out in a 200 cc. transparent thin walled, quartz Erlenmeyer flask provided with a reflux condenser inclined at an angle of 45°. The flask always was placed at a distance of 1 foot below the arc of the Cooper-Hewitt mercury-vapor lamp used during the irradiation of each preparation. 1 gm. of each material was dissolved in 75 cc. of ether, with the exception of dicholesteryl ether, 0.375 gm. of which was dissolved in 75 cc. of ether. The ether solution of each preparation was irradiated for 30 minutes during which time the solution was heated sufficiently on a hot-plate to cause convection currents, thus effecting more uniform irradiation. All preparations so irradiated were tested biologically for antirachitic properties.

Several cholesterol derivatives have been reported as not acquiring antirachitic properties following irradiation with ultraviolet rays. They are cholesterilene (13), dicholesteryl ether (4), cholesteryl chloride (14), dihydrocholesterol (15), cholestene (14), cholestenone (16), and butyl cholesteryl ether (17), whereas cholesteryl acetate (17) can be activated. The data dealing with irradiation as presented in Table I confirm the results obtained by other investigators.

### *Treatment with Reagent*

Cholesterol and its derivatives were treated with sulfuric acid and acetic anhydride in a 50 cc. Erlenmeyer flask stoppered with cotton, by mixing 0.001 mole of a preparation, with the exception of 0.0005 mole in the case of dicholesteryl ether, in glacial acetic acid with 0.002 mole (0.112 cc.) of concentrated sulfuric acid and 0.0025 mole (0.24 cc.) of acetic anhydride. As each mixture was prepared, it was heated immediately at 85–90° on a water bath until a dark gummy residue appeared on the sides of the flask. The greater portion of the acetic acid was removed by exposing it to a current of hot air for 15 minutes, after which the flask was placed in a vacuum to remove the remainder of the solvent.

Several of the preparations used required different amounts of

TABLE I

*Comparative Efficacies of Ultraviolet Irradiation and Sulfuric Acid-Acetic Anhydride in Imparting Antirachitic Activity to Cholesterol and Several of Its Derivatives*

| Preparations treated          | Quantity of preparation fed per rat | Line test* (average plus values and No. of rats) |   |
|-------------------------------|-------------------------------------|--|---|
|                               |                                     | Irradiation                                      | H <sub>2</sub> SO <sub>4</sub> -<br>(CH <sub>3</sub> CO) <sub>2</sub> O |
|                               | mg.                                 |  |   |
| Cholesterol, Wilson, C.F..... | 0.5                                 | 2 (4)  |   |
| “ “ “ .....                   | 1                                   | 3 (4)  |   |
| “ “ “ .....                   | 6                                   |  | 2 (7)   |
| “ recovered†.....             | 500                                 | 3 (4)  |   |
| “ purified‡.....              | 6                                   |  | 3 (7)   |
| “ “ .....                     | 500                                 | 3 (4)  |   |
| Cholesteryl acetate.....      | 12                                  |  | 3 (7)   |
| “ “ .....                     | 150                                 | 4 (4)  |   |
| “ chloride.....               | 6                                   |  | 2 (7)   |
| “ “ §.....                    | 6                                   |  | 3 (4)   |
| “ “ §.....                    | 12                                  |  | 3 (4)   |
| “ “ §.....                    | 500                                 | 0 (10)   |   |
| “ “   .....                   | 6                                   |  | 3 (4)   |
| “ “   .....                   | 12                                  |  | 4 (4)   |
| “ “   .....                   | 500                                 | 0 (6)  |   |
| Cholesterilene.....           | 6                                   |  | 3 (7)   |
| “ .....                       | 6                                   |  | 3 (7)   |
| “ .....                       | 500                                 | 0 (3)  |   |
| Cholestene.....               | 6                                   |  | 2 (7)   |
| “ .....                       | 12                                  |  | 4 (7)   |
| “ .....                       | 500                                 | 0 (2)  |   |
| Butyl cholesteryl ether.....  | 6                                   |  | 2 (7)   |
| “ “ “ .....                   | 12                                  |  | 3 (7)   |
| “ “ “ .....                   | 500                                 | 0 (6)  |   |
| Dicholesteryl ether.....      | 6                                   |  | 1 (7)   |
| “ “ .....                     | 500                                 | 0 (3)  |   |
| Cholestenone.....             | 150                                 | 0 (5)  | 0 (6)   |
| “ .....                       | 500                                 | 0 (7)  |   |
| Dihydrocholesterol.....       | 150                                 | 0 (4)  | 0 (6)   |
| “ .....                       | 500                                 | 0 (7)  |   |

\* A 2 plus value here denotes approximately 5 vitamin D units, United States Pharmacopoeia XI (20). The figures in parentheses denote the number of rats used in each test. Negative and positive control groups of six or more rats were included in each series of comparisons made, and all responded satisfactorily.

† Recovered cholesterol from which nearly all cholesterol provitamin D had been removed.

‡ Prepared by debromination of cholesterol dibromide obtained from recovered cholesterol.

§ Prepared from cholesterol (Wilson, C.F.) known to contain provitamin D.

|| Prepared from purified cholesterol.

acetic acid solvent as well as different lengths of time to complete the activating reaction. These data are summarized in Table II.

Aliquot portions of all preparations treated with sulfuric acid-acetic anhydride were made to volume in carbon tetrachloride solution, to which a small quantity of alcohol had been added. Each was tested subsequently with rats for antirachitic activity. Similarly, known quantities of all preparations were made up to volume in anhydrous ethyl ether, irradiated as described above, and tested for activity.

A specific amount of each solution, based on the original weight of each material tested, was evaporated on a definite quantity of

TABLE II

*Quantity of Acetic Acid Solvent Used and Approximate Reaction Time Required to Induce Activation by Sulfuric Acid-Acetic Anhydride Method*

| Preparation treated             | Quantity of acetic acid used | Reaction time |
|---------------------------------|------------------------------|---------------|
|                                 | cc.                          | hrs.          |
| Cholesterilene.....             | 4                            | 1.5           |
| Cholesterol (Wilson, C.P.)..... | 4                            | 2.0           |
| "    purified.....              | 4                            | 2.0           |
| Cholesteryl acetate.....        | 4                            | 3.5           |
| Cholestene.....                 | 4                            | 3.5           |
| Butyl cholesteryl ether.....    | 4                            | 3.5           |
| Dihydrocholesterol.....         | 4                            | 5.0           |
| Cholesteryl chloride.....       | 8                            | 4.0           |
| Cholestenone.....               | 8                            | 3.5           |
| Dicholesteryl ether.....        | 12                           | 5.0           |

rachitogenic Ration 2965 of Steenbock and Black (18), and tested with rats according to the procedure of McCollum, Simmonds, Shipley, and Park (19) for the line test. The feeding technique employed was of necessity our own modification of that recommended by the United States Pharmacopoeia (20). The comparative potencies induced by ultraviolet irradiation and sulfuric acid-acetic anhydride are presented in Table I.

## DISCUSSION

It can be seen from the data presented in Table I that ordinary cholesterol (Wilson, C.P.) containing provitamin D was rendered

quite active on irradiation; whereas, purified cholesterol became only faintly active when treated similarly. However, they both yielded products, on treatment with sulfuric acid-acetic anhydride, which had essentially the same activity. Likewise, the cholesteryl chlorides, prepared from both ordinary and purified cholesterol, also yielded products which, on treatment with sulfuric acid-acetic anhydride, had the same activity. On the other hand, neither of these chlorides was activatable by irradiation, nor were the derivatives cholesterilene, dicholesteryl ether, cholestene, and butyl cholesteryl ether activatable by irradiation. But on treatment with the activating reagent mentioned above they all acquired antirachitic properties of approximately the same magnitude, when appraised in terms of equal weights of original materials. These data would indicate that the antirachitic substance produced on treatment with sulfuric acid-acetic anhydride was not derived from the provitamin D of cholesterol.

Evidence is presented (Table I) demonstrating that the process of bromination and debromination does not render cholesterol so treated entirely inactivatable by irradiation. This observation is in agreement with that of Bills, Honeywell, and MacNair (21), and confirmed by others.

Dihydrocholesterol and cholestenone were the only preparations included in this study which could not be activated by treatment with sulfuric acid-acetic anhydride. It is to be noted that these are the only preparations which do not have a double bond in the 5, 6 position. The results suggest that a specific relationship probably exists between chemical configuration and chemically activatable cholesterol and cholesterol derivatives.

#### SUMMARY

Ordinary cholesterol (Wilson, c.p.) and purified cholesterol, on treatment with sulfuric acid-acetic anhydride, yield products with essentially the same antirachitic activity, whereas on irradiation with ultraviolet rays they yield a potent and a weak antirachitic product, respectively.

Cholesterilene, dicholesteryl ether, cholesteryl chloride, cholestene, and butyl cholesteryl ether yield antirachitic products on treatment with sulfuric acid-acetic anhydride, but are non-responsive to irradiation. Cholestenone and dihydrocholesterol

do not yield antirachitic products by either treatment. On the other hand, cholesteryl acetate and cholesterol known to contain provitamin D become antirachitically active when subjected to either treatment.

Cholesterol provitamin D is not the precursor of the antirachitic product produced from cholesterol or certain of its derivatives when activated by sulfuric acid-acetic anhydride.

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## THE METABOLISM OF SULFUR

### XXIV. THE METABOLISM OF TAURINE, CYSTEIC ACID, CYSTINE, AND OF SOME PEPTIDES CONTAIN- ING THESE AMINO ACIDS

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The biological relation of taurine and the bile acids, of whose molecules this sulfonic acid is a component, to cystine is uncertain. The conversion of cystine to cysteic acid is readily accomplished in the laboratory, but the decarboxylation of this acid to form taurine, first demonstrated by Friedmann, has been difficult until recently, when White and Fishman (1) established the conditions necessary for this reaction. If taurine has its biological origin from cystine through cysteic acid, and if this is the normal path of catabolism of cystine, ready oxidation of the sulfur of taurine to sulfate, the end-product of cystine metabolism, should be observed. On the other hand, if taurine originates from a specialized path of catabolism of cystine, a reaction which results in a readily available supply of taurine for conjugation with cholic acid to form taurocholic and related acids, the complete oxidation of its sulfur to sulfate would not of necessity be anticipated.

Experimental data have shown that the sulfur of taurine and cysteic acid is not readily oxidized when these acids are administered to the common laboratory animals or to man. The experiments with cysteic acid are few and limited to studies with the dog. The literature concerned with the biological behavior of taurine has been summarized by one of us (2).

It has been shown that the oxidation of the sulfur of cystine does not proceed normally if the amino group is blocked in such a way as to inhibit deamination. In the sulfur-containing acids of the bile, the amino group of the taurine is in combination and does



not react with nitrous acid until liberated by acid hydrolysis. It seems possible that cystine may first conjugate with cholic acid and that the cystine, with its amino group thus blocked, may follow a metabolic path different from that of cystine itself, with the formation of a cysteic acid-cholic acid derivative and taurocholic acid. Taurine and cysteic acid thus in combination might be oxidized when the free acids might fail to be attacked in the animal organism. It is well established that pyrimidines in nucleoside combination, particularly cytosine and, to a lesser degree, uracil and thymine, are more readily and completely catabolized than the free pyrimidines (3). A similar picture might be presented in the oxidation of the biologically significant sulfonic acids, free and in combination.

The present studies are concerned with the distribution of urinary sulfur in the rabbit after the administration of peptides containing taurine or cysteic acid. In these compounds, the amino group is in combination as in the bile acids. It was felt that a study of these peptides might aid in the solution of the problem of the biological origin and significance of these two sulfonic acids.

A similar investigation of the urinary picture after the administration of a type of peptides little studied, the cystinyl peptides (4, 5), is also presented. Since glutathione readily decomposes to give glutamic acid or a derivative and cysteinylglycine, these peptides are of special interest. Moreover, the relation of cystine to the hypoglycemic activity of the polypeptide, insulin, lends additional interest to a study of the behavior of all peptides containing cystine.

#### EXPERIMENTAL

The general procedure and analytical methods were those commonly employed in this laboratory for studies of sulfur metabolism (6), male rabbits serving as the experimental animals. Cystine was prepared from hair; cysteic acid, by oxidation of cystine with bromine (7); taurine, by the method of Marvel and Bailey (8); glycylcysteic acid (9), glycyltaurine (9), and the cystinyl peptides (4), by the methods of White; and diglycylcystine, as described by Abderhalden and Spinner (10). All the compounds were of satisfactory purity, as evidenced by nitrogen and sulfur analyses.

Harris' titration (11) was also employed in the analyses of the peptides.

The compounds, either as such or as the sodium salt in a few cases, were dissolved in 10 to 15 cc. of water for oral administration and in 4 to 10 cc. for subcutaneous injection.

In order to make all the experiments as nearly comparable as possible, when the free sulfur-containing amino acids were to be administered, an amount of amino acid (glycine or alanine) equivalent to that present in the peptides was administered with the sulfur compound. Thus, the amounts of nitrogen and sulfur were the same in all experiments and the variable factor was the presence of the peptide linkage in the case of the peptides. The sulfur compounds were fed in amounts equivalent to 1.0 gm. of cystine (0.267 gm. of sulfur).

#### DISCUSSION

*Cystic Acid, Taurine, and Peptides Containing These Acids (Table I)*—In agreement with the older observations of Salkowski (12), the sulfur of orally administered taurine was oxidized in part to sulfate sulfur. However, since practically all the extra sulfur excreted after the subcutaneous injection of taurine was present in the organic sulfur fraction, it is believed that the oxidation observed after oral administration is to be ascribed to changes effected by the microflora of the intestine rather than to the action of cells of the organism proper. In feeding experiments, two variables, of unknown significance and not easily controllable, are introduced; i.e., rate of absorption and activity of the intestinal microorganisms. These variables have not been considered adequately in many experiments, particularly in the studies of the metabolism of compounds of sulfur. There appears to exist some species difference, since Salkowski (12) and the present workers have observed a considerable degree of oxidation of the sulfur of orally administered taurine in the rabbit, while in the dog (13) and man the oxidation was so slight as to be practically within the limits of experimental error.

No evidence was obtained to indicate that the combination of taurine with glycine as a peptide<sup>1</sup> influenced the oxidation of its

<sup>1</sup> Ackermann (14) was able to isolate, from the urine of the dog, 8 per cent of the taurocyamine (guanyltaurine) fed (20 gm. in 4 days), but no studies of sulfur partition were made.

sulfur, varying degrees of oxidation being observed, as with free taurine, when the peptide was fed; but no oxidation was observed after subcutaneous injection.

It should be noted that in none of our studies here reported was any attempt made to determine whether deamination had occurred

TABLE I

*Distribution of Extra Sulfur in Urine Excreted in 24 Hour Period Immediately Following Administration of Glycine and Taurine, Glycyltaurine, Glycine and Cysteic Acid, and Glycylcysteic Acid*

Extra sulfur values are expressed as per cent of the total sulfur administered, the figures in parentheses showing the percentage distribution of the extra sulfur between sulfate and organic sulfur. The sulfur content of the compounds fed was, in all cases, 0.267 gm. The letters of the Experiment No. are used to designate the individual experimental animals, while the numeral refers to the number of the experiment. All experiments bearing the same letter were carried out with the same animal.

| Experiment No. | Mode of administration | Compounds fed         | Extra sulfur excreted |              |              |
|----------------|------------------------|-----------------------|-----------------------|--------------|--------------|
|                |                        |                       | Total                 | Sulfate      | Organic      |
| M-1            | Oral                   | Glycine, taurine      | 71.6                  | 37.1 (52.0)  | 34.5 (48.0)  |
| M-3            | Subcutaneous           | " "                   | 68.3                  | 2.0 (2.8)    | 66.3 (97.2)  |
| M-18           | "                      | " "                   | 92.3                  | 8.1 (8.8)    | 84.2 (91.2)  |
| M-2            | Oral                   | Glycyltaurine         | 46.5                  | 29.7 (63.9)  | 16.8 (36.1)  |
| M-5            | "                      | "                     | 59.3                  | 35.4 (59.8)  | 23.9 (40.2)  |
| O-16           | "                      | "                     | 52.2                  | 17.6 (33.5)  | 34.6 (66.5)  |
| M-4            | Subcutaneous           | "                     | 80.1                  | -8.6 (-10.8) | 88.7 (110.8) |
| M-19           | "                      | "                     | 98.5                  | 6.2 (6.3)    | 92.3 (93.7)  |
| L-8            | Oral                   | Glycine, cysteic acid | 77.8                  | 21.4 (27.5)  | 56.4 (72.5)  |
| N-10           | Subcutaneous           | " "                   | 50.6                  | -6.8 (-13.4) | 57.4 (113.4) |
| C-32           | "                      | " "                   | 53.6                  | 2.1 (3.9)    | 51.5 (96.1)  |
| L-9            | Oral                   | Glycylcysteic acid    | 41.3                  | 7.2 (26.4)   | 34.1 (73.6)  |
| O-15           | "                      | " "                   | 45.8                  | 12.9 (28.2)  | 32.9 (71.8)  |
| N-11           | Subcutaneous           | " "                   | 69.8                  | 11.5 (16.6)  | 58.3 (83.4)  |
| A-31           | "                      | " "                   | 70.7                  | 25.3 (37.5)  | 45.4 (62.5)  |

or whether a carbamic acid derivative of taurine was excreted. The experiments are concerned solely with the fate of the sulfur-containing group of the molecule.

The results obtained with cysteic acid and its peptide are difficult of exact interpretation. After oral administration of both the

free cysteic acid and the peptide, a significant increase of oxidized (sulfate) sulfur was observed, amounting in three experiments recorded in Table I to slightly more than 25 per cent of the extra total sulfur excreted. This increased urinary excretion of oxidized sulfur was, however, distinctly less than that which was observed after oral administration of taurine or glycyltaurine. After the injection of the free cysteic acid, no increase in the excretion of extra sulfate sulfur was observed. When the peptide was injected, a mode of administration which excludes the intestinal factors previously discussed, some oxidation of sulfur was indicated. It is of interest to compare results of Experiments N-10 and N-11 in which the experimental animal was the same. It has been difficult to calculate exactly the extra sulfur excretion in certain of the glycylcysteic acid experiments, since some increase in the level of total nitrogen excretion was frequently observed in the injection experiments. This should result in an increased sulfur elimination not related to the utilization of the peptide. This objection applies to Experiment A-31. Nevertheless, we feel that there is some evidence of an oxidation of the cysteic acid in peptide linkage apart from changes produced by the intestinal microorganisms. Previous studies of the behavior of cysteic acid, as far as concerns the oxidation of its sulfur, have been limited to experiments with dogs (13).

The similarity of the changes in urinary sulfur distribution, after feeding the peptides and the sulfonic acids, suggested that enzymatic hydrolysis of the peptides might have occurred prior to absorption. The action of enzyme preparations from hog tissue, carboxypolypeptidase (pancreas), "erepsin," and aminopolypeptidase (intestine), and also glycerol extracts of liver and kidney on the two peptides was determined at a pH of 8.0 and at 30°. The activity of the preparations used was demonstrated by control experiments with the usual substrates. No hydrolysis by any of the enzymes of the intestinal tract studied was observed.<sup>2</sup> Glycyltaurine was split readily by the liver preparations, although not so readily as the leucylglycine. In experiments under com-

<sup>2</sup> The experiments with enzymes of the tissues of the hog were carried out by Dr. H. O. Calvery, with the methods of the Waldschmidt-Leitz group. We wish to express our indebtedness for conduct of these experiments and for advice in the other determinations.

parable conditions, 83 per cent cleavage of leucylglycine was observed as compared with a 29 per cent cleavage of glycyltaurine. The cleavages of the substrates by kidney extracts were 75 and 10 per cent, respectively. No significant hydrolysis of glycylcysteine

TABLE II

*Distribution of Extra Sulfur in Urine Excreted in 24 Hour Period Immediately Following Administration of Cystine and Glycine, Cystine and Alanine, Cystinyldiglycine, Diglycylcystine, and Cystinyldialanine*

Extra sulfur values are expressed as per cent of the total sulfur administered, the figures in parentheses showing the percentage distribution of the extra sulfur between sulfate and organic sulfur. The sulfur content of the compounds fed was, in all cases, 0.287 gm. The letters of the Experiment No. are used to designate the individual experimental animals, while the numeral refers to the number of the experiment. All experiments bearing the same letter were carried out with the same animal.

| Experiment No. | Mode of administration | Compounds fed                            | Extra sulfur excreted |             |             |
|----------------|------------------------|--|-----------------------|-------------|-------------|
|                |                        |  | Total                 | Sulfate     | Organic     |
| L-14           | Oral                   | Glycine, <i>l</i> -cystine               | 82.2                  | 70.4 (86.0) | 11.8 (14.0) |
| C-27           | Subcutaneous           | " "                                      | 59.4                  | 52.6 (88.9) | 6.8 (11.1)  |
| C-23           | Oral                   | <i>l</i> -Cystinyldiglycine              | 62.3                  | 48.7 (78.5) | 13.6 (21.5) |
| C-24           | "                      | "  | 73.2                  | 56.3 (77.2) | 16.9 (22.8) |
| C-25           | Subcutaneous           | "  | 58.2                  | 42.2 (72.8) | 16.0 (27.2) |
| A-20           | "                      | "  | 65.3                  | 50.8 (78.0) | 14.5 (22.0) |
| J-33           | Oral                   | Diglycyl- <i>l</i> -cystine              | 64.3                  | 51.8 (80.7) | 12.5 (19.3) |
| M-6            | "                      | "  | 61.1                  | 47.4 (77.8) | 13.7 (22.2) |
| O-17           | "                      | "  | 33.7                  | 31.9 (94.8) | 1.8 (5.2)   |
| J-34           | Subcutaneous           | "  | 56.0                  | 36.1 (69.4) | 19.9 (35.6) |
| M-7            | "                      | "  | 56.7                  | 25.5 (45.1) | 31.2 (54.9) |
| N-12           | "                      | "  | 65.3                  | 37.6 (58.1) | 27.7 (41.9) |
| A-20           | Oral                   | <i>dl</i> -Alanine, <i>l</i> -cystine    | 70.8                  | 45.2 (64.0) | 25.6 (36.0) |
| A-22           | "                      | " "                                      | 105.4                 | 70.3 (66.8) | 35.1 (33.2) |
| A-30           | Subcutaneous           | " "                                      | 53.6                  | 45.9 (85.0) | 7.7 (15.0)  |
| A-21           | Oral                   | <i>l</i> -Cystinyldi- <i>dl</i> -alanine | 82.2                  | 52.9 (64.8) | 29.3 (35.2) |
| A-28           | Subcutaneous           | " "                                      | 70.2                  | 51.9 (74.4) | 18.3 (25.6) |
| C-26           | "                      | " "                                      | 79.3                  | 55.0 (73.2) | 24.3 (26.8) |

acid was obtained with any of the enzyme preparations. Similar results were obtained with preparations from rabbit tissue, although the hydrolysis of both leucylglycine and glycyltaurine was less extensive than with the preparations from hog tissue.

These experiments fail to indicate any opportunity for the hydrolysis of the sulfonic acid peptides in the gastrointestinal canal, except possibly by the action of microbial enzymes. In the tissues, ready hydrolysis of glycyltaurine would appear possible. Whether glycylcysteic acid can undergo cleavage is not apparent from our results.

*Peptides Containing Cystine*—In Table II we present data concerned with the distribution of the extra sulfur excreted in the urine after the oral and subcutaneous administration of *l*-cystine and *dl*-alanine or glycine, diglycyl-*l*-cystine and the cystinyl peptides, *l*-cystinyldiglycine and *l*-cystinyldi-*dl*-alanine. The recoveries of extra total sulfur and the percentage distribution of this extra sulfur are not significantly different, and demonstrate that the sulfur of both types of peptides is as readily oxidized as is the sulfur of cystine itself. There appears to be some indication of the excretion of a greater proportion of oxidized (sulfate) sulfur after the injection of *l*-cystinyldiglycine than after similar administration of diglycyl-*l*-cystine. However, since the experiments were not carried out with the same experimental animal, we do not regard this slight difference as particularly significant.

The data which indicate a ready utilization (as evidenced by oxidation of the sulfur) of peptides containing cystine are in harmony with the only similar study with which we are familiar, that of Abderhalden and Samuely (15). The sulfur of dialanyl-cystine and dileucylcystine was excreted as sulfate sulfur after oral or parenteral administration of these compounds to dogs. Evidence of utilization of cystine present in a peptide (diglycyl-*l*-cystine and di-*dl*-alanyl-*l*-cystine) has also been obtained in growth experiments with white rats by one of us (16). The cystine of the molecule of glutathione may also be utilized for purposes of growth (17). Oxidation of the sulfur of glutathione in the normal organism has also been studied,<sup>3</sup> but the data are not very extensive (18).

In view of the usually accepted relationship of the sulfur-containing component of the insulin molecule to its hypoglycemic action, it is of interest to note that no indication of hypoglycemic

<sup>3</sup> Studies with glycylcysteine (18) indicated a ready oxidation of peptides containing cysteine in the dog.

shock was observed in either injection or feeding experiments, despite the fact that each animal received the equivalent of 1.0 gm. of cystine in combination as a peptide. No determinations of blood sugar were made, however.

The hydrolysis of diglycyl-*L*-cystine by the enzymes of the gastrointestinal tract, previously demonstrated (19), was confirmed and cleavage of *L*-cystinyldiglycine by "erepsin" and aminopolypeptidase (intestine) and carboxypolypeptidase (pancreas) was established. It is evident that there is abundant opportunity for the hydrolysis of the cystine-containing peptides in the intestine.

#### SUMMARY

1. The oral administration of cysteic acid and taurine to rabbits led to a definite but variable increase in the excretion of oxidized (sulfate) sulfur of the urine as well as an increase in the organic sulfur fraction. This oxidation of the sulfur of these two biologically important sulfonic acids is believed to be related to the activity of the microflora of the intestine, since after parenteral (subcutaneous) introduction of these compounds, all the extra sulfur appeared in the organic sulfur fraction.

2. The excretion and distribution of the extra sulfur, after oral or parenteral administration of peptides containing glycine and cysteic acid or taurine, did not differ significantly from those observed after administration of the free sulfonic acids.

3. The sulfur of cystinyl peptides (*L*-cystinyldiglycine and *L*-cystinyldi-*DL*-alanine) was readily oxidized and excreted mainly as oxidized (sulfate) sulfur. No difference between the behavior of the cystinyl peptides studied and that of diglycylcystine in this respect was observed. No reactions resembling insulin shock were observed, although the peptides in amounts equivalent to 1.0 gm. of cystine were fed or injected.

4. *In vitro* studies indicated abundant opportunity for hydrolysis of both types of peptides containing glycine and cystine in the gastrointestinal canal.

5. Similar *in vitro* studies failed to demonstrate any hydrolysis of the peptides containing the sulfonic acids by the enzyme preparation of intestinal tract studied. Glycyltaurine was readily hy-

dolyzed by glycerol extracts of liver and kidney (hog and rabbit), but no cleavage of glycylcysteic acid was observed.

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## THE COLORIMETRIC DETERMINATION OF FREE AND COMBINED CHOLESTEROL\*

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In 1934 Schoenheimer and Sperry (1) published a colorimetric method for the determination of free and total cholesterol based upon the development of the Liebermann-Burchard reaction in an acetic acid solution of the precipitated cholesterol digitonide. These authors claim that by applying the color reaction to a specific chemical compound of cholesterol the possible error introduced by other chromogenic substances is eliminated. The results reported are much more uniform than those obtained by other methods. Sperry (2) found that of 126 samples of blood serum obtained from 91 healthy adults the percentage of free in total cholesterol varied only between 24.3 and 30.1 per cent, with an average of 26.9 per cent, so that the ester (calculated) in total cholesterol varied between 70 and 76 per cent. Unless the use of digitonin as a precipitant be criticized, the Schoenheimer-Sperry procedure seems unquestionably accurate and their standards for normal values would appear to justify general acceptance.

This method, however, is designed for use with a photometer. When an ordinary colorimeter is used the final reading is difficult, since the color produced in the glacial acetic acid is exceedingly faint even when larger quantities of material are taken for analysis.

In this paper we present a new method for the direct colorimetric determination of free cholesterol together with certain modifications of the Bloor (3, 4) and Bloor-Knudson (5) procedures for total and ester cholesterol respectively. The results obtained, as regards the ratio of free and ester cholesterol to total cholesterol, are comparable to those of Schoenheimer and Sperry and, by means of the three determinations, an excellent check is

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provided for the accuracy of each, since obviously the calculated sum of free and ester should equal the total.

In brief, the procedure is as follows: Plasma lipids are extracted with alcohol-ether (3:1) as recommended by Bloor. Total cholesterol is determined on one aliquot of filtrate; Bloor's procedure (4) is followed closely for this. Ester and free cholesterol are determined on a second aliquot, the former by a method similar to that of Bloor and Knudson, except that saponification is introduced, and the latter by analysis of the cholesterol digitonide precipitate by a method to be described.

### *Methods*

#### *Reagents—*

Alcohol, 95 per cent, redistilled over barium hydroxide.

Ether, Baker's c. p. Analyzed.

Petroleum ether, Baker's, range 30–60° (ligroin); unnecessary to redistil.

Potassium hydroxide, 40 per cent aqueous solution.

Digitonin, Hoffmann-La Roche, 0.2 per cent aqueous solution purified and prepared according to Schoenheimer and Sperry (1).

Acetone-absolute alcohol, 1:1.

Chloroform, Squibb's chloroform for anesthesia.

Acetic anhydride, Eastman Kodak Company.

Sulfuric acid, concentrated c. p.

Sulfuric acid, 1:3 dilution.

In all determinations the Mirsky-Bruger (6) conditions for color development have been employed. A colorimeter lamp equipped with daylight glass has been used and all readings made through a red glass filter disk placed on top of the eye-piece of the colorimeter.

*The importance of the quality and freshness of the acetic anhydride and chloroform used cannot be overemphasized.*

### *Procedure*

*Preparation of Plasma Filtrate*—Oxalated blood has been used. Plasma filtrates have been prepared according to Bloor (4), usually 3 or 4 cc. of plasma in 100 cc. of alcohol-ether.

*Determination of Total Cholesterol*—Bloor's procedure (4) has been followed except that in saponifying, 2 or 3 drops of 40 per

cent aqueous potassium hydroxide have been used instead of sodium ethylate.

*Determination of Free and Ester Cholesterol*

*Precipitation of Free Cholesterol*—Transfer exactly 10 or 15 cc. of alcohol-ether filtrate to a 100 cc. beaker and evaporate to dryness, preferably at a temperature less than 60°. It is convenient to use an oven regulated at 40–50°. Rapid evaporation at a higher temperature is permissible if care is taken to avoid overheating, which may produce a yellow color.

Extract the dry residue into a 30 cc. beaker with two 1.5 cc. and two 1 cc. portions of acetone-alcohol, filtering through a small plug of fat-free cotton inserted in the neck of a funnel. Add 2 cc. of digitonin solution, mix the contents by gently rotating the beaker, and allow precipitation to take place for at least 4 hours or preferably overnight.

*Extraction and Determination of Ester Cholesterol*—Place the beaker containing the white flocculent precipitate of cholesterol digitonide on the steam bath and quickly evaporate to complete dryness. Extract the ester with warm petroleum ether as directed in the Bloor-Knudson procedure (5), *taking care that the larger part of the precipitate remains in the beaker and that no precipitate is lost.* Place the funnel used in filtering the ester over the beaker containing the cholesterol digitonide precipitate and set aside for determination of the free fraction.

To the petroleum ether extract add 3 drops of 40 per cent potassium hydroxide. Mix well and allow saponification to take place at a temperature of about 75–85°, preferably on a sand bath. Evaporation of the petroleum ether and the characteristic appearance of the syrupy saponified residue usually take place in 15 to 20 minutes. With most blood filtrates complete saponification with no loss of ester will take place under these conditions.<sup>1</sup> After saponification is complete proceed as in the determination of total cholesterol.

*Determination of Free Cholesterol*—Allow 1 cc. of hot glacial acetic

<sup>1</sup> Saponification of the ester may take place satisfactorily in alcohol-ether under conditions analogous to those employed for the total determination. We have found, however, that frequently values 5 to 10 per cent lower than the calculated are obtained by this procedure. At the present time no explanation can be offered as to the reason for the apparent loss.

acid to drop slowly through the funnel into the beaker containing the thoroughly dry cholesterol digitonide precipitate previously set aside. If much precipitate is on the cotton, it is well to dislodge the plug with a pair of small forceps and shake it gently, allowing the particles to drop on the funnel, thus insuring the transfer of all the cholesterol digitonide to the beaker. Warm the beaker for a few seconds to complete solution of the precipitate and then transfer quantitatively the acetic acid solution of the cholesterol digitonide to a round bottomed 15 cc. centrifuge or culture tube, two portions of 0.5 cc. each of hot acetic acid being used to wash out the beaker.

Fit the tube with a cork stopper which has a water pump connection and a groove on one side through which a stirring rod may pass. Provision is thus made for a gentle air current to be drawn through the tube. Place the tube on a sand bath (temperature 150–160°), attach to the water pump, and with gentle suction evaporate the acetic acid *almost* to dryness without charring. This is usually accomplished in 5 to 10 minutes. *When just a trace of acid remains but before evaporation is complete* remove the tube from the suction apparatus, add 5 cc. of petroleum ether, and stir vigorously with the glass rod while the petroleum ether is boiling. The free digitonin appears as a white or yellowish white precipitate and the free cholesterol quantitatively passes into the petroleum ether. Centrifuge and quantitatively decant the petroleum ether containing the free cholesterol into a 50 cc. Erlenmeyer flask. Wash the digitonin precipitate twice with 2 to 3 cc. portions of warm petroleum ether and add the washings to the original extract. Determine the free cholesterol in this petroleum ether extract exactly as in the total and ester determination. A standard containing 0.3 mg. of cholesterol may be used if desired instead of the regular 0.5 mg. standard or the 0.5 mg. standard may be used and the colorimeter set at 5 instead of 10.

*Comment*—We prefer the aqueous solution of digitonin as directed, although check determinations have been made with an alcoholic solution of digitonin, either in an acetone-alcohol medium or in petroleum ether as recommended by Kirk, Page, and Van Slyke (7). In the procedure as outlined, 1 mg. of free cholesterol is completely precipitated and no ester is held back. If a

blood filtrate is encountered in which more than 1 mg. of free cholesterol may be expected in the quantity of filtrate usually taken, a smaller volume of filtrate must be used.

Centrifuge tubes may be used rather than beakers for the precipitation of the digitonide. Although this technique has the advantage of lessened manipulation and transference, the disadvantages are numerous. Evaporation to dryness of the solution in a centrifuge tube is necessarily slow and not always satisfactory. To extract the ester completely, several washings are necessary, with a corresponding number of centrifugations, which is more laborious and time-consuming than filtration as outlined.

TABLE I

*Comparison of Total Cholesterol (Bloor's Method) and Analysis of Digitonide after Saponification*

The results are expressed in mg. per 100 cc. of plasma.

| Filtrate used | Bloor's procedure | Analysis of digitonide |
|---------------|-------------------|------------------------|
| Normal.....   | 210               | 212                    |
| Diabetic..... | 206               | 203                    |
| " .....       | 396               | 392                    |
| " .....       | 396               | 387                    |

### Results

*Method for Total Cholesterol. Recovery of Known Amounts—*Recovery of total cholesterol from solutions containing known amounts of free cholesterol and cholesterol acetate has been shown to be complete.

| Calculated<br>mg. | Found<br>mg. |
|-------------------|--------------|
| 0.610             | 0.615        |
| 0.610             | 0.600        |
| 0.684             | 0.675        |

*Comparison of Results Obtained by Bloor's Procedure and by Analysis of Digitonide Precipitate after Saponification—*To ascertain whether the results obtained for total cholesterol by the Bloor procedure are comparable to those obtained by a procedure analogous to the Schoenheimer-Sperry method, the following experiment was carried out: Four alcohol-ether filtrates were saponified and petroleum ether extracts prepared in the usual

TABLE II  
*Recovery of Free and Ester Cholesterol from Known Solutions with and without Addition of Blood Filtrate*

| Free cholesterol  |  |   | Ester cholesterol   |  |                                     |
|---|--|---|---|--|-------------------------------------|
| Contents of solution analyzed                             | Theoretical in volume analyzed<br>mg.                    | Amount recovered<br>mg.                                     | Contents of solution analyzed                             | Theoretical in volume analyzed<br>mg.                              | Amount recovered<br>mg.             |
| Free cholesterol  | 0.495  | 0.495   | Cholesterol acetate                                       | 0.45   | 0.44                                |
| "   | 0.495  | 0.485   | "   | 0.67   | 0.67                                |
| " and cholesterol acetate                                 | 0.370  | 0.350   | "   | 0.681  | 0.665                               |
| Free cholesterol, cholesterol acetate, and blood filtrate | 0.12 (Filtrate)<br>0.34 (Known cholesterol)<br>—<br>0.46 | 0.47<br>0.43<br>0.46<br>0.48<br><u>0.43</u><br>0.45 Average | Cholesterol acetate, free cholesterol, and blood filtrate | 0.33 (Filtrate)<br>0.45 (Known cholesterol acetate)<br><u>0.78</u> | 0.76<br>0.74<br><u>0.75</u> Average |
| "   | 0.14 (Filtrate)<br>0.495 (Known cholesterol)             | 0.62<br>0.61<br><u>0.615</u> Average                        | "   | 0.30 (Filtrate)<br>0.45 (Known cholesterol acetate)<br><u>0.75</u> | 0.72<br>0.72<br><u>0.72</u> Average |

manner. In each case two aliquots of the petroleum ether extract were evaporated to dryness. The residue from the first was extracted with chloroform and total cholesterol determined according to the regular Bloor procedure. The residue from the second was extracted with acetone-alcohol, digitonin added, and total cholesterol determined upon the precipitated cholesterol digitonide as outlined above under the determination of free cholesterol. The results obtained (Table I) indicate that the two methods yield identical results and that the determination of total cholesterol by analysis of the definite chemical compound, cholesterol digitonide, gives values differing in no way from those obtained by

TABLE III

*Duplicate Determinations of Free and Ester Cholesterol*

The results are expressed in mg. per 100 cc. of plasma.

| Filtrate No. | Free cholesterol | Average | Ester cholesterol | Average | Total cholesterol | Calculated total |
|--------------|------------------|---------|-------------------|---------|-------------------|------------------|
| 1            | 92               | 90      | 153               | 151     | 250               | 241              |
|              | 88               |         | 149               |         |                   |                  |
| 2            | 74               | 74      | 195               | 195     | 269               | 269              |
|              | 74               |         | 195               |         |                   |                  |
| 3            | 68               | 66      | 156               | 153     | 224               | 219              |
|              | 64               |         | 149               |         |                   |                  |
| 4            | 56               | 55      | 159               | 158     | 213               | 213              |
|              | 54               |         | 156               |         |                   |                  |
| 5            | 68               | 68      | 209               | 205     | 281               | 273              |
|              | 68               |         | 201               |         |                   |                  |

Bloor's method of direct determination on the extract after saponification.

*Method for Free and Ester Cholesterol*—By the procedures outlined, recovery of both free and ester cholesterol from known solutions with and without the addition of blood filtrate has been shown to be complete (Table II). Duplicate determinations of free and ester cholesterol can be obtained with a variation of not more than 5 per cent (Table III). The calculated sum of these two values equals the total cholesterol figure obtained (after saponification) by the Bloor procedure and the percentage of ester or free cholesterol in the total cholesterol lies usually within the range considered normal by Sperry (Table IV).



TABLE IV

*Total, Free, and Ester Cholesterol in Blood Plasma*

The results are expressed in mg. per 100 cc. of plasma unless noted otherwise.

| Filtrate No. | Total saponified | Free | Ester saponified | Per cent free in total | Per cent ester in total   |                       | Calculated total of free and ester |
|--------------|------------------|------|------------------|------------------------|---------------------------|-----------------------|------------------------------------|
|              |                  |      |                  |                        | Based on calculated value | Based on actual value |                                    |
| 1*           | 156              | 40   | 120              | 26                     | 74                        | 77                    | 160                                |
| 2            | 194              | 50   | 136              | 26                     | 74                        | 70                    | 186                                |
| 3            | 283              | 71   | 211              | 25                     | 75                        | 75                    | 282                                |
| 4            | 240              | 70   | 168              | 29                     | 71                        | 70                    | 238                                |
| 5            | 215              | 55   | 166              | 26                     | 74                        | 77                    | 221                                |
| 6            | 234              | 61   | 165              | 26                     | 74                        | 70                    | 226                                |
| 7*           | 152              | 44   | 106              | 29                     | 71                        | 70                    | 150                                |
| 8            | 225              | 65   | 160              | 29                     | 71                        | 71                    | 225                                |
| 9*           | 205              | 62   | 145              | 30                     | 70                        | 70                    | 207                                |
| 10*          | 145              | 41   | 100              | 29                     | 71                        | 69                    | 141                                |
| 11*          | 245              | 62   | 178              | 25                     | 75                        | 73                    | 240                                |
| 12           | 240              | 66   | 173              | 28                     | 72                        | 72                    | 239                                |
| 13           | 259              | 77   | 181              | 30                     | 70                        | 70                    | 258                                |
| 14*          | 144              | 44   | 99               | 31                     | 69                        | 69                    | 143                                |
| 15*          | 212              | 58   | 142              | 27                     | 73                        | 66                    | 200                                |
| 16           | 269              | 62   | 205              | 23                     | 77                        | 76                    | 267                                |
| 17           | 257              | 68   | 185              | 26                     | 74                        | 72                    | 253                                |
| 18           | 226              | 58   | 166              | 26                     | 74                        | 73                    | 224                                |
| 19*          | 137              | 42   | 91               | 31                     | 69                        | 66                    | 133                                |
| 20           | 224              | 66   | 153              | 29                     | 71                        | 68                    | 219                                |
| 21           | 239              | 59   | 178              | 25                     | 75                        | 75                    | 237                                |
| 22           | 239              | 64   | 166              | 27                     | 73                        | 69                    | 230                                |
| 23*          | 157              | 46   | 109              | 29                     | 71                        | 69                    | 155                                |
| 24*          | 178              | 50   | 118              | 28                     | 72                        | 65                    | 168                                |
| 25           | 248              | 62   | 183              | 25                     | 75                        | 74                    | 245                                |
| Minimum      |                  |      |                  | 23                     | 69                        | 65                    |                                    |
| Maximum      |                  |      |                  | 31                     | 77                        | 77                    |                                    |
| Average      |                  |      |                  | 28                     | 73                        | 71                    |                                    |

In developing our methods a considerable part of the work has been done on blood filtrates from diabetic patients. The filtrates from normal individuals which we have analyzed would, however, justify the conclusion that the ratio of free and ester cholesterol in these diabetic filtrates may be regarded as normal. It may be observed in Table IV that the average percentages for free and *calculated* ester compare very closely with those given by Sperry and previously quoted. There is a greater variation in the actual ester value, although the mean deviation of the difference between calculated and actual total values is only 1.9 per cent.

\* Non-diabetic blood.

## DISCUSSION

*Use of Oxalated Plasma*—Potassium oxalate has been used as an anticoagulant for the sake of uniformity in hospital procedure. Although the results obtained may be appreciably lower than on heparinized plasma (8), any error thus introduced will be constant.

*Completeness of Extraction*—The completeness of extraction by the Bloor procedure has been amply substantiated (9-11).

TABLE V

*Comparison of Direct and Saponified Total Cholesterol Values with Direct and Saponified Ester Values*

The results are expressed in mg. per 100 cc. of plasma.

| Filtrate | Total cholesterol |         | Ester cholesterol |         | Free cholesterol | Calculated totals         |                       |
|----------|-------------------|---------|-------------------|---------|------------------|---------------------------|-----------------------|
|          | Saponified        | Direct* | Saponified        | Direct† |                  | Free and saponified ester | Free and direct ester |
| C. B.    | 248               | 265     | 182               | 200     | 62               | 244                       | 262                   |
| E. B.    | 224               | 230     | 153               | 168     | 66               | 219                       | 234                   |
| S. H.    | 259               | 312     | 181               | 231     | 77               | 258                       | 308                   |
| D. F.    | 328               | 387     | 237               | 312     | 88               | 325                       | 400                   |
| L. N.    | 257               | 297     | 185               | 215     | 68               | 253                       | 283                   |
| N. F.    | 162               | 197     | 107               | 141     | 53               | 160                       | 194                   |
| H. N.    | 173               | 208     | 121               | 165     | 43               | 164                       | 208                   |
| "        | 180               | 202     | 126               | 148     | 57               | 183                       | 206                   |
| T. B.    | 307               | 386     | 209               | 298     | 104              | 313                       | 402                   |
| N. F.    | 151               | 181     | 96                | 132     | 59               | 155                       | 191                   |

\* *Direct total cholesterol* we have determined by evaporating a measured portion of alcohol-ether filtrate to dryness, extracting the residue with warm petroleum ether, filtering the extract through fat-free cotton, and determining as in the regular procedure.

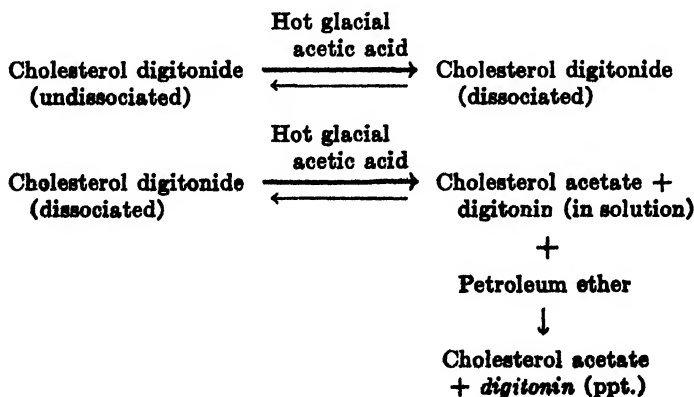
† *Direct ester cholesterol* we have determined by applying the regular technique to the petroleum ether filtrate obtained after precipitation of the digitonide.

*Modifications of Bloor-Knudson Method*—The Bloor-Knudson procedure has been modified in two ways. First, free cholesterol has been precipitated in an acetone-alcohol medium at room temperature as recommended by Schoenheimer and Sperry. More consistent results have been obtained by this technique than with the direct Bloor-Knudson method. Second, saponification of the ester has been introduced. This step is essential if the procedure for total cholesterol involves saponification. Table V

shows clearly that a valid ratio between ester and total cholesterol can be obtained only if saponification is either included or omitted in both determinations. The factor responsible for the well recognized discrepancy (4, 12) which may exist between saponified and unsaponified total cholesterol values is obviously associated with the ester fraction. The nature of the chromogenic factor is not clear. Reinhold's suggestion (13) that the discrepancy may be explained by the difference in velocity of the color reaction of free and ester cholesterol scarcely seems tenable unless his exact conditions for color development are reproduced. At any rate, in our experience saponification makes no appreciable difference with known pure solutions of either free or ester cholesterol (p. 677) provided overheating is avoided.

*Determination of Free Cholesterol*—The method proposed for free cholesterol is based on the dissociation of cholesterol digitonide in glacial acetic acid. Schoenheimer and Dam (14) found recovery of cholesterol from a pyridine solution of the digitonide to be only 85 per cent from the first ether extraction. We have quantitatively recovered cholesterol (Table II) by using hot glacial acetic acid as the solvent and extracting with boiling petroleum ether. Extraction is always incomplete, however, giving low results if evaporation of the acetic acid proceeds to complete dryness.

In explaining the reaction which takes place in the procedure as outlined, the following hypothesis seems plausible: In the presence of hot acetic acid dissociated cholesterol digitonide is



converted into cholesterol acetate and digitonin, the latter being precipitated upon the addition of petroleum ether, as shown in the diagram on p. 682.

Since under the conditions for color development used a known amount of cholesterol yields the same color whether free or as the acetate, the supposition that free cholesterol is actually determined as the acetate is justified.

| Substance analyzed                    | Weight taken | Weight recovered        |       |
|---------------------------------------|--------------|-------------------------|-------|
|                                       | mg.          | mg.                     | mg.   |
| 1. Free cholesterol                   | 0.495        | 0.490<br>0.485<br>0.500 | 0.492 |
| 2. Cholesterol as cholesterol acetate | 0.681        | 0.685                   |       |
| 3. " "                                | 0.770        | 0.770<br>0.765          | 0.768 |

The methods outlined should give little trouble to the person familiar with the Bloor technique for total cholesterol. The ester determination is simpler than that for free cholesterol but, with a little experience, the latter determination is not difficult. If not used routinely, the free cholesterol determination is strongly recommended as a check on the ester cholesterol determination if the ratios found vary widely from the accepted normal range.

#### SUMMARY

1. The Bloor colorimetric method for total cholesterol has been shown to check a method analogous to the Schoenheimer-Sperry procedure in which total cholesterol is determined from analysis of the digitonide precipitate obtained after saponification.

2. A colorimetric method for free cholesterol, based upon an analysis of the digitonide precipitate, and comparable with Bloor's method for total cholesterol, is outlined.

3. A modification of the Bloor-Knudson method for ester cholesterol is suggested.

4. Evidence is presented indicating that the factor responsible for the higher total cholesterol values usually obtained on unsaponified blood filtrates is associated with the ester fraction.

5. Sperry's normal ratios of free and ester cholesterol to total cholesterol have been substantiated.

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## FACTORS INFLUENCING THE STABILITY OF INSULIN

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Before any given lot of insulin can be marketed, its potency and stability must meet certain requirements. Stability is arbitrarily determined by subjecting a sample of insulin to a "heat test." This test consists of exposing the carefully standardized insulin to a temperature of 52° for 10 days. The potency of the "heated" material is then compared either with a standard insulin or with a sample of the same preparation not exposed to the heat test. As a rule insulin thus treated loses some of its physiologic activity, but if there should be a considerable loss, *e.g.* 15 per cent or more, then such a preparation is not considered suitable for general use. The purpose of the heat test is to insure all insulin preparations for stability under adverse conditions. The causes of the loss of potency of the "heated" insulin have not, hitherto, been established. Traces of certain metals, particularly copper and iron, were found in some of the unstable preparations; it was therefore assumed that the presence of these metals might be a factor affecting deterioration either upon prolonged standing at room temperature or upon subjection to a heat test.

The deterioration of insulin upon standing is a problem of considerable importance not only to the manufacturer but to the physician, and in particular, to the diabetic patient. Thus it was deemed advisable to secure data on this problem. For this purpose it was considered desirable to study a preparation of insulin low in its ash content and practically free of such metals as copper, iron, and zinc.

This paper deals with (1) a method for the preparation of an insulin low in ash and free of copper, iron, and zinc; (2) the effect of temperature on the stability of such a preparation; and (3)

the effect of temperature on such a preparation to which known amounts of copper, iron, and zinc had been added.

### *Method of Preparation*

The method for the preparation of an insulin low in its ash content and free of copper, iron, and zinc is as follows:

To 400 cc. of commercial insulin 100 units per cc. (40,000 units) 100 cc. of 1 M ammonium acetate and 60 cc. of acetone are added. The reaction is adjusted to about pH 6.5, and the mixture is allowed to stand overnight and filtered or centrifuged. The reaction of the clear filtrate is lowered to about pH 5.0 and allowed to stand in the refrigerator for 3 or 4 days, centrifuged, and the precipitate dissolved in dilute sulfuric acid to pH 2 or 2.5 and brought to a volume of 500 cc. The acidified solution is treated with 4 volumes of 95 per cent denatured ethyl alcohol and allowed to stand overnight. Any precipitate formed is removed by filtration through a hardened filter paper. The volume of the clear alcoholic filtrate is brought to 3.5 liters by the addition of absolute alcohol followed by 3.5 liters of ether. The mixture is allowed to stand for 2 days in a refrigerator. The clear alcohol-ether is siphoned off and the precipitate centrifuged.

The alcohol-ether precipitate is dissolved in lactic acid and filtered. The reaction of the solution is carefully adjusted to the isoelectric point with 1 N ammonium hydroxide. This process of precipitation with lactic acid and ammonium hydroxide is repeated twice. After the third precipitation, the precipitate is again taken up with sulfuric acid to pH 2 and to a volume of 500 cc., and 4 volumes of alcohol are added. Any precipitate formed is removed by filtration and the alcohol-soluble fraction is precipitated with alcohol-ether in the manner already described. The alcohol-ether precipitate is taken up in 100 cc. of distilled water and dissolved by the use of lactic acid. Then 50 cc. of 10 per cent trichloroacetic acid are added. The trichloroacetic acid precipitate formed is centrifuged and taken up in 90 per cent ethyl alcohol and filtered. An equal volume of absolute alcohol is added, followed by twice its volume of ether along with 1 cc. of 1 M ammonium acetate. The addition of ammonium acetate is very helpful in securing a complete flocculation of the insulin protein. After standing overnight the precipitate thus formed is

carefully centrifuged and washed twice with ether and dried in a vacuum.

Since the main object is to secure a preparation of low ash content, no attempt was made to secure any data on the amount of insulin lost. This process was followed on two different lots with the following results.

| Lot No. | Potency                | Ash             | Nitrogen        | Copper, iron,<br>zinc |
|---------|------------------------|-----------------|-----------------|-----------------------|
|         | <i>units per 1 mg.</i> | <i>per cent</i> | <i>per cent</i> |                       |
| 1       | 22                     | 0.09            | 13.71           | None                  |
| 2       | 22                     | 0.096           | 13.72           | "                     |

#### *General Procedure*

*Assay*—The above preparation of low ash, zinc-free insulin was assayed according to the method of Sahyun and Blatherwick (1).

*Stock Dilution*—100 units per 1 cc. of this insulin were prepared as follows: A known amount of the dry insulin powder was accurately weighed and dissolved in 0.1 N hydrochloric acid. Enough of this acid was added to dissolve the insulin and to bring about a reaction of approximately pH 3. As a preservative, 0.1 cc. of tricresol was added to every 100 cc. This stock solution of 100 units of insulin in each cc. was kept in a refrigerator and later used in the various experiments described in this paper.

*Metals*—Separate solutions containing 1 mg. of the following metals were prepared: copper, iron, and zinc. Accurately weighed amounts of the purest metals available were dissolved in either hydrochloric or sulfuric acid and made up to a concentration of 1 mg. in each cc. Copper was dissolved in sulfuric acid; iron and zinc were dissolved in hydrochloric acid. The amount of each metal used is given in each experiment.

*Temperature*—In order to test the stability of insulin, the various samples were kept in an incubator carefully adjusted to about 52°. Samples were removed from the incubator once every week and made up to the proper dilution suitable for assay and kept in the refrigerator. This procedure was essential, as only one sample could be tested each day.

*Blood Sugar*—Sahyun's modification of Folin and Wu's method (2) was employed for blood sugar determination.



## EXPERIMENTAL

*Experiment 1*—25 cc. of the low ash, metal-free insulin, 100 units per cc., were introduced into each of a series of 50 cc. volumetric flasks. Sample A was used as a control. Sample B contained 2.5 mg. of copper, Sample C 2.5 mg. of iron, and Sample D 2.5 mg. of zinc. The contents of the flasks were next diluted to the 50 cc. mark with redistilled water, thoroughly mixed, stoppered, and kept in an incubator regulated at 52°. Once every week 2 cc. were removed from each sample for assay. This experiment lasted for 9

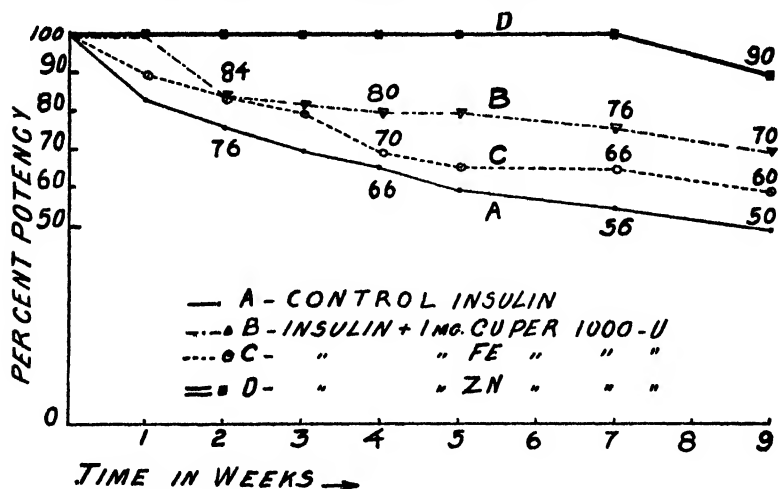


FIG. 1. The effect of temperature (52°) on the potency of insulin with and without added metals.

weeks and the results of the weekly assay of each sample, expressed in terms of per cent potency, are shown in Fig. 1.

It is apparent from the results shown in Fig. 1 that the control Sample A showed a loss of about 17 per cent of its potency 1 week after incubation at 52° and a 50 per cent loss of potency at the end of 9 weeks incubation. On the other hand, and contrary to expectation, the samples to which copper (Sample B) and iron (Sample C) had been added showed a more gradual decrease in potency than did the control sample. Finally, to our great surprise the sample containing 1 mg. of zinc per 1000 units showed a remarkable stability with no detectable decrease in potency after

incubation for at least 7 weeks and with only 10 per cent decrease at the end of 9 weeks incubation at 52°.

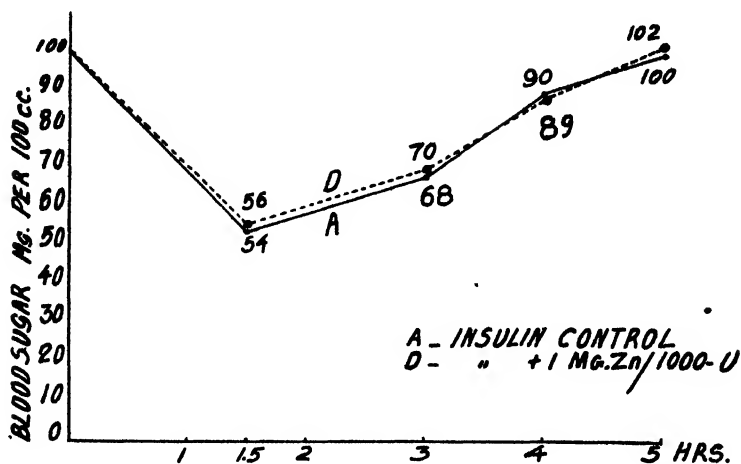


FIG. 2. The effect of insulin before incubation, with and without added zinc, on the blood sugar of rabbits.

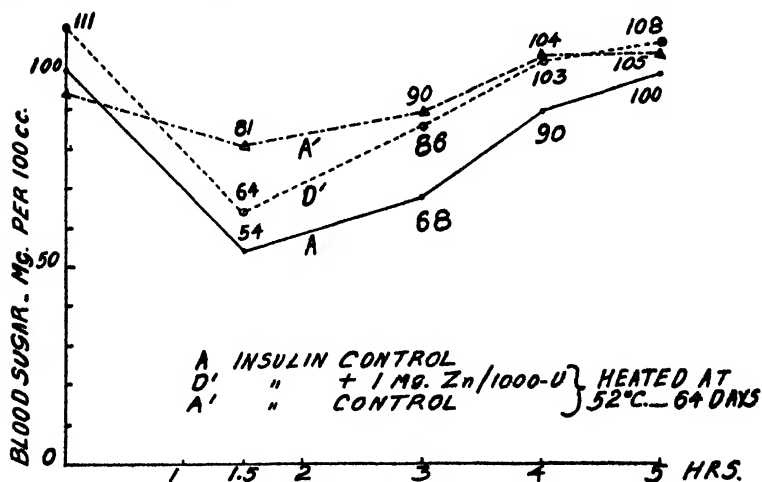


FIG. 8. The effect of "heated" insulin, with and without added zinc, on the blood sugar of rabbits.

**Experiment 2**—This experiment consisted of a comparative study of the effect of insulin, prior to its incubation at 52°, with

and without the addition of zinc, on the blood sugars of twenty-four rabbits. The amount of zinc added was 1 mg. of the metal to every 1000 units of insulin. In each instance the insulin was diluted so that 1 cc. contained 2 units, and 0.5 unit per kilo was injected subcutaneously into each rabbit after a 24 hour fast. Samples of blood were withdrawn from the marginal ear vein at 0, 1.5, 3, 4, and 5 hours after injection. During the 1st week, twelve of the rabbits received the control insulin and the other

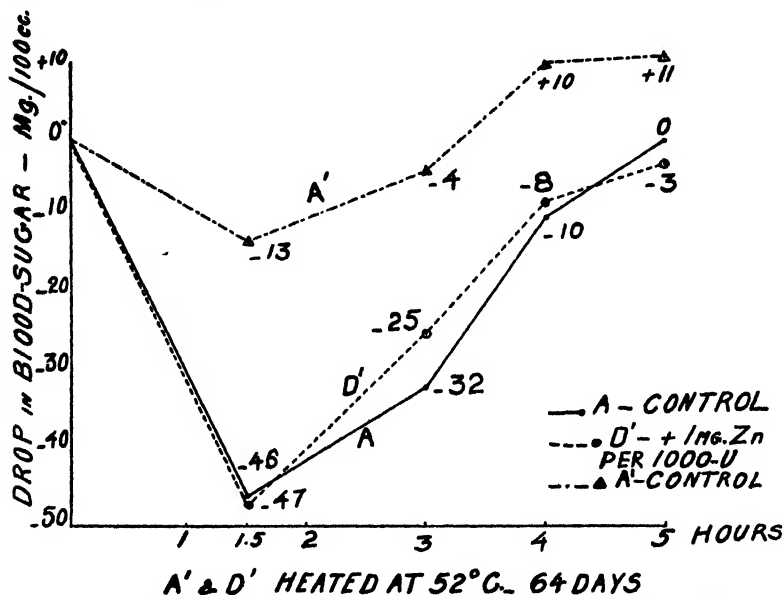


FIG. 4. The effect of "heated" insulin, with and without added zinc, on the blood sugar of rabbits. The results express the actual differences between the blood sugars at 1.5, 3, 4, and 5 hours and the initial figures.

twelve received the insulin to which zinc had been added; during the following week the same animals were used, but those previously receiving the control insulin were given insulin plus zinc and those receiving insulin plus zinc were given control insulin. The averages of blood sugars at the intervals indicated were next plotted, as shown in Fig. 2.

This experiment demonstrates that the addition of 1 mg. of zinc per 1000 units neither increases the efficiency nor tends to prolong the duration of the hypoglycemic action of insulin on the blood sugars of normal fasting rabbits.

**Experiment 3**—In this experiment a comparison was made between the effects of the "unheated" control against the "heated" insulin (at 52° for 9 weeks) with and without added zinc. The procedure described in Experiment 2 was followed on the same animals. The averages of blood sugars are shown in Fig. 3.

In studying the averages of blood sugars shown in Fig. 3 it is observed that the averages for the three initial blood sugars are at different levels. Since the amount of insulin subcutaneously administered was below the convulsive dose, its effectiveness in lowering the blood sugar at a given time might well be represented by the actual differences between the blood sugar at that given time and the initial blood sugar. Thus if the initial blood sugars are 111, 100, and 94 mg. per cent and the blood sugars at 1.5 hours are 64, 54, and 81 mg. per cent respectively, the actual decreases will be 47, 46, and 13 mg. respectively. Values thus obtained were calculated and plotted as shown in Fig. 4.

#### SUMMARY

A method for the preparation of an insulin low in ash and free of copper, iron, and zinc is described. Such a preparation was shown to be unstable when incubated at 52° for at least 1 week. At the end of 9 weeks incubation at this temperature the insulin lost 50 per cent of its physiologic activity.

When 1 mg. of copper or 1 mg. of iron was added to every 1000 units and incubated at 52°, not only was no greater loss in the physiologic activity of the insulin observed but in fact deterioration seemed to proceed at a slower rate (see Fig. 1).

The sample of insulin to which 1 mg. of zinc per 1000 units was added appeared to be quite stable at a temperature of 52° for 7 weeks. At the end of 9 weeks incubation at 52° the sample lost only 10 per cent of its physiologic activity.

Insulin to which 1 mg. of zinc per 1000 units was added did not cause either a delayed onset or a greater duration of its hypoglycemic effect (see Fig. 2).

Further work on the influence of ions of different metals on the stability of insulin is in progress.

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# THE PREPARATION OF HEMOGLOBIN IN A DRY AND ACTIVE STATE\*

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It is well known that crystals of oxyhemoglobin are changed by drying into a form which retains little or no activity with oxygen. Bohr (1) has called the inactive material  $\beta$ -hemoglobin, and Hufner (2) has shown it to be a mixture of methemoglobin with variable small amounts of oxyhemoglobin. Heidelberger (3), describing his method of crystallizing oxyhemoglobin, cautions against allowing the crystals to become dry if loss in activity is to be avoided. Van Slyke, Hastings, Heidelberger, and Neill (4) state, "If dried, even at high vacuum and in the cold, hemoglobin is almost completely inactivated, although in solubility and color it still resembles oxyhemoglobin." Barcroft (5), referring to his own experience with it and that of Bayliss and Parsons, calls such dried material inactive hemoglobin.

In our initial unsuccessful attempts (which included the use of various organic solvents) to improve the activity of dried oxyhemoglobin we noted that methemoglobin formation could be largely if not completely avoided by reducing the hemoglobin before drying. We are now able to define conditions for preparing reduced hemoglobin in a dried and stable condition without significant loss of oxygen capacity.

## EXPERIMENTAL

*Hemoglobin Solutions and Crystals*—Oxalated or defibrinated blood of the dog, pig, ox, or human is centrifuged, the plasma

\* A preliminary report was made before the American Society of Biological Chemists (*Proc. Am. Soc. Biol. Chem.*, 8, lxxiii (1936); *J. Biol. Chem.*, 114 (1936)).

removed as closely as possible, and the cells washed in the centrifuge four or five times with cold 0.9 per cent NaCl. The washed cells are then treated in one of the following ways: (a) chilled in an ice bath and an equal volume of ice-cold distilled water added (the mixture is then extracted, with centrifuging, three times with ice-cold c.p. anhydrous ether<sup>1</sup> to complete laking and remove stroma); (b) diluted, laked with alumina cream, and centrifuged to remove stroma; (c) after addition of sufficient cold distilled water to lake completely, centrifuged at high speed to remove any débris; (d) diluted with 0.9 per cent NaCl without laking; (e) as in (c), followed by dialysis against distilled water in the cold room.

Crystals of dog hemoglobin were prepared by a procedure which we have described (6).

There is no indication that the method of preparing the hemoglobin solution has any effect upon the activity of the dried residue provided the preliminary treatment does not in itself cause inactivation.

*Method of Drying*—Drying is accomplished by vacuum distillation at 38°. The apparatus consists of a glass drying chamber connected by pressure tubing through a 3-way stop-cock to a flask containing concentrated H<sub>2</sub>SO<sub>4</sub>, which, in turn, is connected through a mercury manometer to a Cenco Hyvac pump. The inlet tube to the flask extends below the surface of the acid to facilitate absorption of water vapor. For small samples of hemoglobin, up to 40 ml. of solution, the drying chamber is a 1 liter round bottom Pyrex flask with ground-in stop-cock. For larger samples, up to 200 ml., a large flat bottom desiccator is used.

In drying small samples, the hemoglobin solution is placed in the drying chamber, the system closed, and the pump started. Initial foaming is controlled through stop-cock regulation by allowing the pressure in the drying chamber to fall slowly. As reduction proceeds foaming diminishes, and pressure in the chamber is then allowed to fall to that of the rest of the system. The chamber is shaken vigorously by hand to assure as complete reduction of the hemoglobin as possible. The sample, which is cold

<sup>1</sup> Anhydrous ether was used, as it contained the least amount of impurities of any grade of ether we were able to purchase. Most grades of ether contain impurities which convert hemoglobin to methemoglobin.

when placed in the chamber, is kept cold during the reduction of the hemoglobin by the rapid evaporation of the water, thus minimizing methemoglobin formation at this most critical stage. The chamber is now placed in a water bath at 38°, and the acid-absorbing flask in an adjoining bath at room temperature. Distillation proceeds rapidly and the final pressure is too low to be measured accurately on our manometer. Both chamber and flask are rocked by a motor-driven mechanism. Thus the hemoglobin solution spreads and dries in a thin film, while agitation of the acid in the absorbing flask increases its efficiency.

If crystals are to be dried, they must be suspended in sufficient water to dissolve completely as they are reduced, before appreciable drying occurs.

When larger samples are to be dried, the desiccator is connected while empty, rocking is started, and the whole system is evacuated. The hemoglobin solution is then slowly admitted to the evacuated chamber through a capillary tube whose tip is drawn to a very blunt point to prevent clogging by the accumulation of dried hemoglobin on the outside of the capillary. The hemoglobin solution enters the chamber as a fine, hair-like stream which strikes the bottom with considerable velocity and spatters to the sides of the chamber. Reduction is practically instantaneous, foaming scarcely occurs, and the small droplets dry almost at once. However, the drying capacity of our system is not sufficient to prevent the accumulation of some liquid and a considerably longer time is required to dry the larger samples under these conditions. We have not been able to get quite as high activity or uniform drying as with the smaller samples.

In either procedure drying is continued until flakes of dry hemoglobin begin to pull away from the sides of the chamber. If the chamber is now removed from the bath and struck a sharp blow with the hand, a considerable amount of the hemoglobin is detached from the walls. The time required for drying is about 20 minutes for the smaller samples and up to 3 hours for the largest samples.

The dried hemoglobin is handled in one of two ways, with effects upon properties which will be discussed later. (a) The sample is removed from the drying chamber and ground to a powder in a mortar without protection from oxygen of the air. Portions may



be dissolved immediately and analyzed, and other portions sealed *in vacuo* in glass ampules. (b) An alternative procedure, which gives consistently higher activities of the dried hemoglobin, is to connect the drying chamber by pressure tubing to a tonometer containing somewhat more distilled water than will be used to dissolve the sample. The tonometer, in turn, is attached to a vacuum line and the water completely deaerated. As the tonometer is shaken to facilitate removal of air, evaporation of water causes it to become quite cool. During this evacuation water travels back to the closed stop-cock of the drying chamber in an unbroken column. The desired quantity of water is now admitted to the chamber and, with shaking, the sample dissolves rapidly, forming a solution of reduced hemoglobin. So little gas is present that the solution gives a metallic click and practically no foam when shaken. Air is now admitted and the hemoglobin is readily converted on shaking into oxyhemoglobin.

#### *Analytical Methods*

All gasometric analyses were made in the Van Slyke manometric blood apparatus. Total pigment was determined by the method of Van Slyke and Hiller (7) or spectrophotometrically by the procedure of Drabkin and Austin (8), or by both methods, and frequently by the use of undried control samples. After our samples are dissolved and oxygenated, the combined experimental error in oxygen capacity and total pigment estimations does not exceed  $\pm 1$  per cent. Data on Samples 130-b and 135-b (Table II) are representative of repeated checks upon our experimental error. Approximately the first one-fourth of the samples was analyzed for active hemoglobin by the carbon monoxide capacity method of Van Slyke and Hiller (9), and the remainder by the oxygen capacity method of Van Slyke and Neill (10). A few samples were determined by both methods with excellent agreement in results. A number of samples analyzed by the oxygen capacity method were also analyzed with the spectrophotometer (Bausch and Lomb), with a modification of the procedure of Austin and Drabkin (11).

In the spectrophotometric analysis of a mixture of oxyhemoglobin and methemoglobin as outlined by Austin and Drabkin, three values or sets of values are required: the extinction coeffi-

cients of the mixture at  $\lambda$  630, 575, 560, and 540  $m\mu$ ; the pH of the mixture; the concentration of total pigment. The modification of their procedure which we have employed eliminates determination of the pH and is, we believe, slightly more accurate. Three aliquots of the mixture to be analyzed are pipetted into volumetric flasks of the same capacity. The sample in Flask 1 is diluted, 5 ml. of phosphate buffer of pH 7.38 are added, and the mixture made to volume with distilled water. The other two samples are diluted and quantitatively converted to methemoglobin by potassium ferricyanide. To the mixture in Flask 2 are now added 5 ml. of the buffer, pH 7.38, and sufficient distilled water to bring to volume. Potassium cyanide is added to Flask 3 to form cyanmethemoglobin, and the mixture is diluted to volume with water. Concentrations in all cases are those given by Austin and Drabkin (11). Total pigment is determined in Solution 3. Extinction coefficients at  $\lambda$  630, 575, 560, and 540  $m\mu$  are measured for Solutions 1 and 2, which have the same pH and the same ionic strength, except for the effect of the small amount of ferricyanide in Solution 2. Calculations are made by the method of Austin and Drabkin (11). Agreement between values for active pigment by the oxygen capacity method and the spectrophotometric method was good. Obviously, the experimental error in the spectrophotometric method increases rapidly when one component of a mixture is present in disproportionately small amount.

Residual moisture of the samples was obtained by drying to constant weight in air at 105°.

#### *Properties of Dried Hemoglobin*

Dried hemoglobin appears bright red in color when thin films are viewed by transmitted light. It has a darker red color when powdered. It is freely soluble in distilled water, physiological saline, and phosphate buffers. While no attempt has been made to determine the maximum solubility of our preparations, we have frequently made solutions which had an oxygen capacity up to 14 volumes per cent.

In solutions of the dried hemoglobin there is a small amount of difficultly soluble material which, however, is present only in traces when crystalline hemoglobin is dried. That this material is not

hemoglobin or a hemoglobin derivative is indicated by the following facts. Its removal from the solution by centrifuging does not affect the values for total pigment. When separated and suspended in 0.9 per cent NaCl, it dissolves slowly, forming a solution which has the appearance of plasma from slightly hemolyzed blood. When plasma is dried by our procedure, the proteins

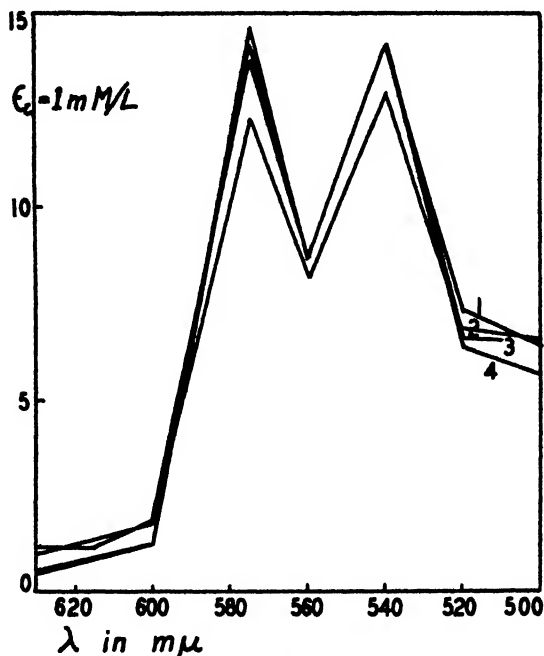


FIG. 1. Spectrophotometric curves of dried hemoglobin dissolved in the presence of air. Curve 1, pig hemoglobin, 91.2 per cent active; Curve 2, ox hemoglobin, 79.2 per cent active; Curve 3, ox hemoglobin, 96.0 per cent active; Curve 4, pig hemoglobin, 92.1 per cent active.

redissolve rather slowly, behaving as does the residue from our hemoglobin samples. We conclude, therefore, that this residue is plasma proteins which are not completely removed even by five washings of the erythrocytes with 0.9 per cent NaCl.

When the dried samples are dissolved without exposure to air, the solutions have the characteristic color of reduced hemoglobin; when air is admitted, the color changes to the bright red of oxy-

hemoglobin. The solutions may be reduced and oxygenated repeatedly by alternate use of vacuum and admission of air. In solution the pigment is readily converted by ferricyanide to methemoglobin and the latter, by cyanide, to cyanmethemoglobin.

That the hemoglobin has not been appreciably altered by drying is indicated by the following facts: (a) In solution it combines re-

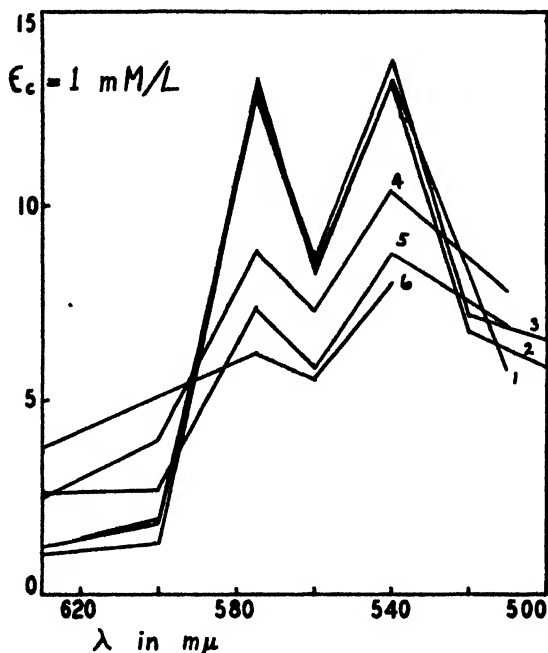


FIG. 2. Spectrophotometric curves of Sample 93. Curve 1 from Ampule 93-c, activity 82.1 per cent; Curve 2, Ampule 93-e, activity 80.1 per cent; Curve 3, Ampule 93-f, activity 85.9 per cent; Curves 4, 5, and 6, samples from these same ampules exposed to air for varying periods of time before analysis.

versibly with oxygen and with carbon monoxide; (b) crystals of oxyhemoglobin which appear to be identical with those obtained from fresh dog blood may be obtained by usual methods from solutions of dried dog hemoglobin; (c) Figs. 1 and 2, which show spectrophotometric curves of solutions of dried hemoglobin, are typical of mixtures of oxyhemoglobin and methemoglobin.

Dried hemoglobin in powdered form takes up oxygen rapidly from the air and more rapidly at higher oxygen tensions. When samples are thus prepared for sealing in ampules, as the pressure in the ampules is reduced to a few mm., the powder begins to seethe and sputter from the trapped and possibly adsorbed air. To

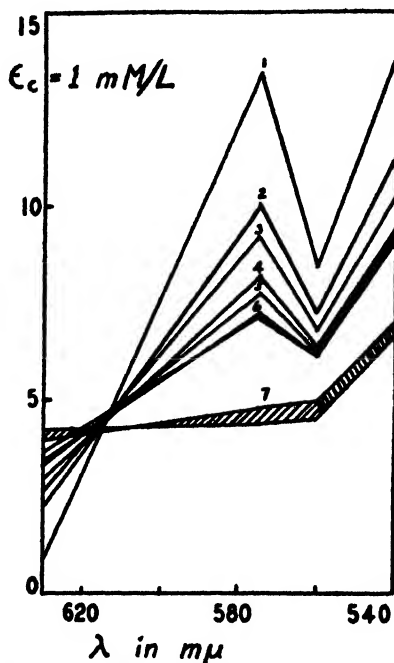


FIG. 3. Curves showing the conversion of dried hemoglobin to methemoglobin when exposed to air. Curve 1, exposed 0 hours, 88.4 per cent activity; Curve 2, 24 hours, 55.5 per cent activity; Curve 3, 46.5 hours, 44.7 per cent; Curve 4, 69 hours, 34.0 per cent; Curve 5, 93 hours, 30.3 per cent; Curve 6, (a) 171 hours, 26.9 per cent, (b) 171 hours, 25.3 per cent; Curve 7 is a composite of all control methemoglobin curves. The variations in these curves are due in part to some variation in pH and in ionic strength.

avoid having the sample carried out of the ampule it is necessary to place relatively small amounts of the powder in the tubes, plug these with cotton, and continuously tap the tubes during evacuation.

The velocity of oxygen uptake by dried hemoglobin and its

relation to methemoglobin formation are being studied, and we expect to report later in more detail upon these behaviors. That methemoglobin is formed under these conditions is shown in Figs. 2 and 3. The activity data and spectrophotometric curves were obtained upon powdered samples whose oxygen uptake had been followed in Warburg respirometers. The gas phase in the respirometers was air at a temperature of 38°, and the time intervals were as noted in Fig. 3. Extinction coefficients were measured at four wave-lengths.

Agreement of data for activity of our dried preparations by both gasometric and spectrophotometric analyses suggests that methemoglobin is the principal, if not sole, pigment contaminant, the spectrophotometric data being calculated on the assumption that this is so.

No further methemoglobin formation and loss of activity occur in dried and powdered samples when they are sealed in evacuated glass ampules over periods of many months.

#### DISCUSSION

In developing a method for drying reduced hemoglobin with retention of its reversible activity towards oxygen we have prepared and analyzed 135 individual samples. Some of these, *e.g.* Samples 93 and 110 (Table III), have been analyzed as many as six times at varying intervals after drying. Methods used in determining active and total pigment have been checked repeatedly against each other, both on dried samples and on undried controls, with excellent agreement.

Data for the first 129 samples are summarized in Table I in the order of activity. All of these samples were dissolved in contact with air. Under such conditions activities approaching 100 per cent have been obtained only with very small samples which in drying were uniformly distributed over the sides of the chamber in a very thin film; these dissolved within 1 or 2 minutes to form rather dilute solutions. With larger samples, delayed solution, and higher concentration, exposure to air causes appreciable loss in activity.

We have had more difficulty in obtaining high activities with dog hemoglobin than with ox, pig, or human when the oxygen-inactivating factor was not excluded at critical stages of prepara-

tion and solution of the samples. Dog hemoglobin, of the four varieties, is the most readily crystallized and, likewise, is slower to dissolve after drying; more methemoglobin is thus formed. In earlier attempts to dry dog hemoglobin crystals very thick suspensions of the crystals were used in expectation of shortening the drying time. However, drying occurred very quickly only on the surface and the underlying crystals were not reduced. Such preparations were invariably low in or devoid of activity. Other samples of the same crystals when suspended in sufficient distilled water to dissolve completely upon reduction, but before appreciable drying had taken place, gave activities up to 93 per cent. It is apparent that rapid drying of a mixture of reduced hemoglobin and oxyhemoglobin greatly accelerates methemoglobin forma-

TABLE I  
*Dried Samples Dissolved in Presence of Air*

| No. of samples | Activity        |
|----------------|-----------------|
|                | <i>per cent</i> |
| 2              | 98-100          |
| 8              | 95- 98          |
| 23             | 90- 95          |
| 33             | 85- 90          |
| 28             | 75- 85          |
| 35             | 10- 75          |

tion. Indeed, conversion to methemoglobin is so rapid, and under proper conditions so complete, as to furnish a method for preparing methemoglobin without the use of an oxidizing agent other than oxyhemoglobin.

Such considerations, we believe, explain the failure of Van Slyke and his coworkers to obtain dry, active hemoglobin preparations. Likewise, the necessity of dissolving the dried reduced hemoglobin samples in an oxygen-free atmosphere to avoid loss of activity is explained. With oxygen present the first hemoglobin to dissolve is converted to oxyhemoglobin and this in contact with the as yet undissolved reduced hemoglobin forms methemoglobin. These conditions are the reverse of those in the process of drying thick, crystalline suspensions, where reduced hemoglobin in solution is in contact with solid oxyhemoglobin; but the result is the same.

Not as much methemoglobin is formed in dissolving samples as in drying them because of the lower temperature in the first instance and the shorter time required for complete solution than for complete drying.

In Table II are seen the greatly improved activities of samples dissolved in the reduced state and oxygenated only after solution was complete. Sample 130 is of especial interest, since data are given for the activity of the dried sample dissolved *in vacuo*, an undried control, and a portion of the same hemoglobin dried exactly as the *in vacuo* sample but dissolved in the presence of air.

TABLE II  
*Dried Samples Dissolved in Distilled Water in Vacuo*

| Sample No. | Species | Active pigment       | Total pigment        | Activity        |
|------------|---------|----------------------|----------------------|-----------------|
|            |         | <i>vol. per cent</i> | <i>vol. per cent</i> | <i>per cent</i> |
| 130-a      | Pig     | 11.82                | 12.32                | 96.0            |
| 130-b*     | "       | 16.80                | 16.97                | 99.0            |
| 130-c†     | "       | 13.89                | 15.59                | 89.1            |
| 131        | "       | 14.01                | 14.31                | 97.9            |
| 132        | Dog     | 6.56                 | 6.71                 | 97.8            |
| 133        | Human   | 7.66                 | 7.85                 | 97.6            |
| 134        | "       | 9.08                 | 9.28                 | 97.7            |
| 135-a      | Ox      | 9.56                 | 9.76                 | 97.9            |
| 135-b*     | "       | 13.84                | 13.73                | 100.8           |

\* Undried control samples as a check upon the accuracy of the methods used.

† A sample of a pig blood, No. 130, dried in exactly the same way as Sample 130-a but dissolved in the presence of air instead of *in vacuo*.

A 10 per cent loss in activity due to methemoglobin formation when the sample is dissolved in contact with air is to be compared with a 3 per cent loss in activity when air was excluded during solution. Similar comparisons are shown for Samples 110 and 111 (Table III).

While our method of drying yields preparations which appear completely dry, which can be easily powdered to a fine dust, and which gain weight when exposed to air at room temperature, on heating in air at 105° there is a loss in weight of 3 to 6 per cent. We have taken this to represent residual water content of the dried samples and in the earlier part of our work we believed that



this value might have some relation to activity. Inspection of Fig. 4 shows that no such relationship is apparent. We are still under the impression, however, that residual moisture content of the dried samples may affect the rate of methemoglobin formation upon their exposure to air; our data on this point are as yet too scanty to justify a definite conclusion.

It was observed early that dried samples become completely inactive when exposed to air for a few days. The data of Table III

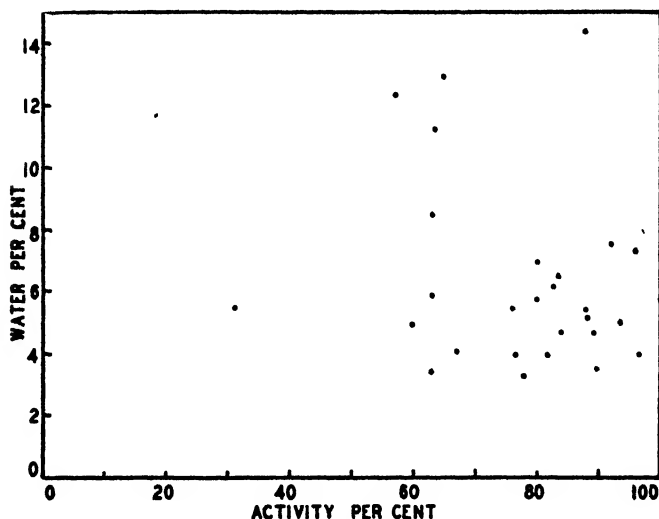


FIG. 4. The moisture content of these samples was varied intentionally to determine whether there is any relation of moisture to activity.

demonstrate the stability of the same or similar preparations when kept for long periods in evacuated ampules. These data confirm the supposition that methemoglobin is not formed unless oxygen is present. In Table III the letter following the sample number designates the ampule of that particular sample. Variations in activity of the hemoglobin from different ampules of the same sample are probably due to inequalities in sampling and to differences in methemoglobin formation during solution of the several portions. Certainly four of the samples dissolved *in vacuo* show a higher activity than portions of the same samples dissolved

in contact with air. The generally lower activities of the sealed samples represented in Table III are to be attributed to the rela-

TABLE III  
*Activity of Samples Sealed in Ampules*

| Sample No. | Species | Age of sample | Activity        |
|------------|---------|---------------|-----------------|
|            |         | <i>days</i>   | <i>per cent</i> |
| 20         | Dog     | 370           | 63.1            |
| 21         | Ox      | 29            | 78.6            |
| 22         | "       | 28            | 61.5            |
| 54-a       | Pig     | 42            | 83.5            |
| 54-b       |         | 185           | 87.5            |
| 93-a       | Ox      | 3             | 87.1            |
| 93-b       |         | 18            | 83.8            |
| 93-c       |         | 55            | 82.1            |
| 93-d       |         | 73            | 76.4            |
| 93-e       |         | 105           | 80.7            |
| 93-f       |         | 133           | 83.4            |
| 103-a      | "       | 0             | 70.7            |
| 103-b      |         | 196           | 60.3            |
| 103-c      |         | 196           | 70.2            |
| 103-d      |         | 196           | 59.4            |
| 108-a      | "       | 14            | 79.8            |
| 108-b      |         | 161           | 84.0            |
| 108-c      |         | 193           | 80.0            |
| 108-d      |         | 236           | 80.7            |
| 108-e      |         | 236           | 80.3            |
| 109-a      | Pig     | 51            | 73.3            |
| 109-b      |         | 51            | 78.5            |
| 110-a      | Ox      | 161           | 76.1            |
| 110-b      |         | 161           | 80.4            |
| 110-c      |         | 233           | 80.8            |
| 110-d*     |         | 233           | 89.1            |
| 110-e*     |         | 234           | 87.7            |
| 110-f*     |         | 234           | 85.6            |
| 111-a      | "       | 161           | 61.7            |
| 111-b      |         | 161           | 65.7            |
| 111-c      |         | 234           | 71.1            |
| 111-d*     |         | 234           | 73.6            |

\* Samples dissolved *in vacuo* instead of in the presence of air.

tively large amounts of hemoglobin which were dried in one lot to provide a sufficient quantity for repeated analyses. Further-

more, some of these lots were prepared before we fully appreciated some of the factors which affect activity.

We are indebted to Dr. T. P. Nash, Jr., for suggesting this problem and for his kind encouragement.

#### SUMMARY

1. A method is described for drying reduced hemoglobin with little or no loss in activity.
2. Some properties of the dry hemoglobin and solutions thereof are described.
3. In the dried state reduced hemoglobin may be preserved apparently indefinitely *in vacuo* without change in activity.

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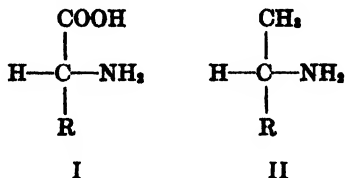
# THE CORRELATION OF THE CONFIGURATION OF NORLEUCINE TO 2-AMINOHEXANE

By P. A. LEVENE AND SERGE MARDASHEW\*

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New York)

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The present communication deals with the correlation of the configurations of aliphatic  $\alpha$ -amino acids of type (I) with the corresponding amines of type (II).



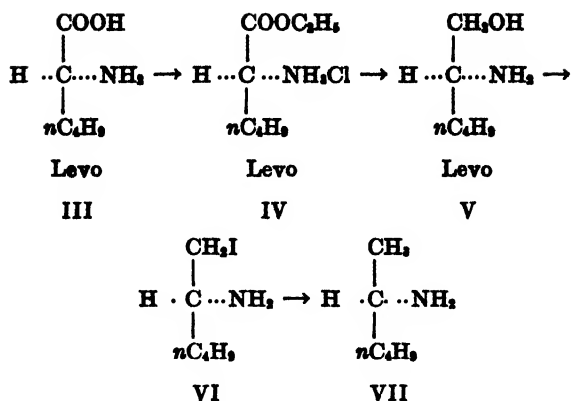
For this purpose the  $\alpha$ -acetamino- and the  $\alpha$ -benzaminocaproic ethyl esters were reduced in alcoholic solution by means of metallic sodium to the 1-hydroxy-2-aminohexane, which, in turn, was heated in a solution of hydrogen iodide in glacial acetic acid, and the product derived in each case was reduced to the amine by means of hydrogen in the presence of Raney's catalyst. The amine was isolated in the form of its hydrochloride. The rotation of this salt, however, was too small to be read with sufficient accuracy. It was therefore transformed into the benzoate. From unpublished work of this laboratory it is known that the free amine and its benzoate rotate in the same direction.

The set of reactions leading from the amino acid to the amine is shown in formulæ (III) to (VII).

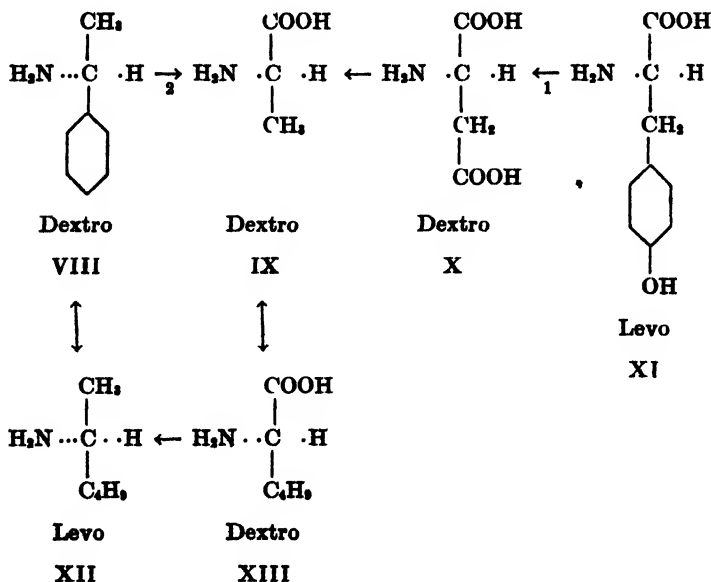
In an earlier publication it was shown<sup>1</sup> that levo-tyrosine and therefore also levo-phenylalanine are configurationally related to dextro-aspartic acid and hence to dextro-alanine. Hence the

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<sup>1</sup> Levene, P. A., and Mardashew, S., *J. Biol. Chem.*, **117**, 179 (1937).



configurations of the two aromatic amino acids likewise are correlated to the aliphatic amines. On the other hand, Leithe<sup>2</sup> has correlated the configuration of 1-amino-1-phenylethane to that of alanine. It is therefore possible to correlate all the normal aliphatic amino acids and the two aromatic amino acids to both the aliphatic and other aromatic amines. Their relationships can be seen from formulæ (VIII) to (XIII).



<sup>2</sup> Leithe, W., *Ber. chem. Ges.*, **64**, 2827 (1931).

From this it follows that the configurationally related aliphatic amine (XII) and phenylated amine (VIII) rotate in opposite directions. It is noteworthy that a similar relationship exists between the corresponding carbinols.<sup>3</sup> Also the relationship of the direction of rotation of substances (XII) and (XIII) is similar to that of the corresponding hydroxyl derivative.

#### EXPERIMENTAL

*$\alpha$ -Bromo-*n*-Caproic Acid*—*n*-Caproic acid boiling at 148–157° was converted into the bromo acid in the usual manner, and the latter was resolved as the strychnine salt until an acid was obtained having  $[\alpha]_D^{20} = -37.97^\circ$ . It distilled at 106–107° at 4 mm. pressure.

*d-Norleucine*—50.0 gm. of the above acid were dissolved in 10 volumes of a 40 per cent solution of ammonia in water, and the solution was allowed to stand at room temperature overnight. The solution was then concentrated under reduced pressure to dryness. The residue was taken up in alcohol and benzene and again concentrated to dryness under reduced pressure. The operation was repeated several times. The residue was extracted with boiling methyl alcohol and the extract concentrated. Yield, 31 gm. The amino acid crystallized. It was recrystallized from water to obtain a substance of higher optical activity.

3.931 mg. substance: 7.920 mg. CO<sub>2</sub> and 3.518 mg. H<sub>2</sub>O

5.140 " " : 0.490 cc. N<sub>2</sub> at 30° and 755 mm.

C<sub>6</sub>H<sub>13</sub>O<sub>2</sub>N. Calculated. C 54.96, H 9.92, N 10.71

131.1 Found. " 54.94, " 10.01, " 10.65

$$[\alpha]_D^{20} = \frac{-1.95^\circ \times 100}{1 \times 11.7} = -16.7^\circ \text{ (in 20\% HCl)}$$

*Benzoyl-d-Norleucine Ethyl Ester*—The ester hydrochloride was prepared in the usual way and had the following composition.

4.625 mg. substance: 8.228 mg. CO<sub>2</sub> and 3.880 mg. H<sub>2</sub>O

13.803 " " : 10.221 " AgCl

C<sub>20</sub>H<sub>25</sub>O<sub>4</sub>NCl. Calculated. C 49.07, H 9.25, Cl 18.15

195.6 Found. " 48.51, " 9.38, " 18.32

<sup>3</sup> Levene, P. A., and Harris, S. A., *J. Biol. Chem.*, **113**, 55 (1936).

To a solution of 30 gm. of the above substance in 60 cc. of 99.8 per cent ethyl alcohol slightly less than the theoretical amount of sodium ethylate was added. The sodium chloride was removed by filtration, and to the filtrate 20 gm. of benzoyl disulfide were added. The solution was refluxed on the water bath for 2 hours. On standing overnight in the refrigerator, the benzoyl derivative settled out in crystalline form. It was recrystallized from dilute alcohol and once more from ether, when it crystallized in long prismatic needles. M.p.  $76^{\circ}$  (uncorrected). Yield, 36 gm.

4.796 mg. substance: 12.000 mg.  $\text{CO}_2$  and 3.410 mg.  $\text{H}_2\text{O}$   
 $\text{C}_{15}\text{H}_{21}\text{O}_2\text{N}$ . Calculated. C 68.44, H 7.98  
 263.2 Found. " 68.23, " 7.96

$$[\alpha]_D^{25} = \frac{+0.91^{\circ} \times 100}{1 \times 10.26} = +8.87^{\circ} \text{ (in ethyl alcohol)}$$

*Reduction of Benzoyl Ester of d-Norleucine Ethyl Ester to Norleucinol*—15.0 gm. of the ester were dissolved in 120 cc. of specially prepared anhydrous ethyl alcohol. The solution was transferred to a 3-neck flask provided with a mechanical stirrer and a reflux condenser. To the solution 10 gm. of sodium-metal were added in small portions in the course of 40 minutes. When the sodium was completely dissolved, the heating was continued an additional 30 minutes. The reaction product was diluted with 2 liters of water and the solution was partially neutralized with 36 gm. of sulfuric acid. The base was steam-distilled into a dilute solution of hydrochloric acid. The solution was concentrated to dryness and the crystalline residue recrystallized from acetone. Yield, 3.3 gm. from two runs.

4.203 mg. substance: 7.200 mg.  $\text{CO}_2$  and 4.005 mg.  $\text{H}_2\text{O}$   
 $\text{C}_8\text{H}_{15}\text{ONCl}$ . Calculated. C 46.90, H 10.45  
 153.5 Found. " 46.71, " 10.66

$$[\alpha]_D^{25} = \frac{-0.18^{\circ} \times 100}{1 \times 18.82} = -0.95^{\circ} \text{ (in water)}$$

*2-Aminohexane Hydrochloride*—1.5 gm. of the above norleucinol in a solution of 15 cc. of glacial acetic acid saturated with hydrogen iodide gas were placed in a sealed tube and heated for 4

hours at 125°. The reaction product was diluted with water and decolorized with sulfur dioxide gas. The sulfuric acid formed in the reaction was removed quantitatively, the filtrate from barium sulfate concentrated to nearly dryness, taken up in methyl alcohol, and reduced by means of hydrogen in the presence of Raney's catalyst. The reaction product was taken up in a dilute solution of sodium hydroxide and the base steam-distilled into dilute hydrochloric acid. The solution was concentrated to dryness and the crystalline dry residue was transferred from the flask by means of ether. M.p. 102–103° with decomposition.

4.692 mg. substance: 9.065 mg. CO<sub>2</sub> and 4.905 mg. H<sub>2</sub>O

C<sub>8</sub>H<sub>13</sub>NCl. Calculated. C 52.36, H 11.63  
137.5 Found. " 52.68, " 11.69

In view of the fact that the optical rotation of the substance was too small to be measured accurately, it was transformed into the benzoyl derivative.

*2-Aminoheptane Benzoate*.—The above hydrochloride was converted into the benzoyl derivative by the Schotten-Baumann method. It was recrystallized three times from ether and petroleum ether. M.p. 82° (uncorrected).

4.742 mg. substance: 13.200 mg. CO<sub>2</sub> and 3.910 mg. H<sub>2</sub>O

C<sub>13</sub>H<sub>19</sub>NO. Calculated. C 76.03, H 9.33  
205.2 Found. " 75.89, " 9.22

$$[\alpha]_{5780}^{25} = \frac{+0.16^{\circ} \times 100}{2 \times 10} = +0.8^{\circ} \text{ (in alcohol)}$$





## DOES TRYPSIN INACTIVATE UREASE?

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(Received for publication, December 16, 1936)

The action of various proteolytic enzymes upon urease has been studied by Zakowski (1), Tauber (2), Tauber and Kleiner (3), Waldschmidt-Leitz and Steigerwaldt (4, 5), and Sumner and his collaborators (6-8). We consider that the protein nature of urease has been conclusively settled and do not wish to renew discussion of this subject. But we wish to point out the inaccuracy of certain statements made by Tauber and by Tauber and Kleiner in regard to the action of trypsin upon urease.

Tauber (2) reported that, if crystalline urease is incubated with trypsin at 37° at pH 7.0, the activity entirely disappears within 3 days. A control under the same conditions showed no loss of urease activity.

After the publication of the paper by Waldschmidt-Leitz and Steigerwaldt (4) in which the claim was made that urease was not inactivated by trypsin, Tauber felt that an explanation of his results was demanded. He and Kleiner (3) repeated the experiments of Waldschmidt-Leitz and Steigerwaldt and confirmed them absolutely. They then showed that the original findings of Tauber were due to the fact that gum arabic was present. As presented in Table I, the experiments of Tauber and Kleiner demonstrate that crystalline urease mixed with gum arabic is almost completely inactivated by Fairchild's or Difco trypsin in 3 days at 37°, while in the absence of gum arabic incubation with trypsin brings about no loss of urease activity whatsoever. Likewise, urease is found to be perfectly stable in the presence of boiled trypsin and gum arabic. Crystalline trypsin is shown to be an exceedingly powerful reagent, since it totally inactivated urease in gum arabic within 8 hours.

While gum arabic was found to have a most remarkable effect in rendering urease subject to attack by trypsin, Tauber and Kleiner observed that, if the urease were allowed to stand with trypsin for 15 hours before adding gum arabic, there was no inactivation of the urease for 3 days. Hence they concluded that trypsin forms a compound with urease which is irreversible.

The results of Tauber and Kleiner would be most startling if true, but we have been unable to verify them. We have repeated the digestions exactly as described by Tauber and Kleiner except

TABLE I  
*Results of Tauber and Kleiner*

| Experiment No. |  | Percentage inactivation when incubated at 37° for |        |        |
|----------------|--|---|--------|--------|
|                |  | 1 day   | 2 days | 3 days |
| 1              | Fairchild's trypsin, urease in gum arabic        | 58  | 86     | 95     |
| 2              | Fairchild's boiled trypsin, urease in gum arabic | 0   | 0      | 0      |
| 3              | Fairchild's trypsin and urease                   | 0   | 0      | 0      |
| 4              | Difco trypsin, urease in gum arabic              | 52  | 83     | 90     |
| 5              | " boiled trypsin, urease in gum arabic           | 0   | 0      | 0      |
| 6              | Difco trypsin and urease                         | 0   | 0      | 0      |
| 7              | Crystalline trypsin, urease in gum arabic        | (Complete in 8 hrs.)                              |        |        |
| 8              | Boiled crystalline trypsin, urease in gum arabic | 0   | 0      | 0      |
| 9              | Crystalline trypsin and urease                   | (None within 3 days)                              |        |        |

that we employed recrystallized urease and in somewhat larger amount than they did.<sup>1</sup> Some of our results are shown in Fig. 1. Here it will be observed that the urease loses its activity at nearly the same rate whether in the presence of gum arabic and active trypsin (Curve I), in the presence of active trypsin alone (Curve II), or in the presence of gum arabic and boiled trypsin (Curve III). It is quite certain that gum arabic exerts no influence upon the rate of inactivation of urease by trypsin. Our curves show that the rate of inactivation of the urease is slightly more rapid in the

<sup>1</sup> We have employed only Fairchild's trypsin.

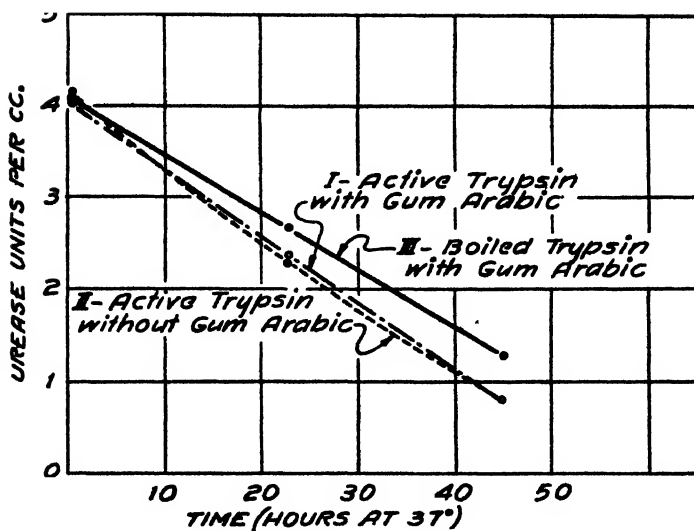


FIG. 1

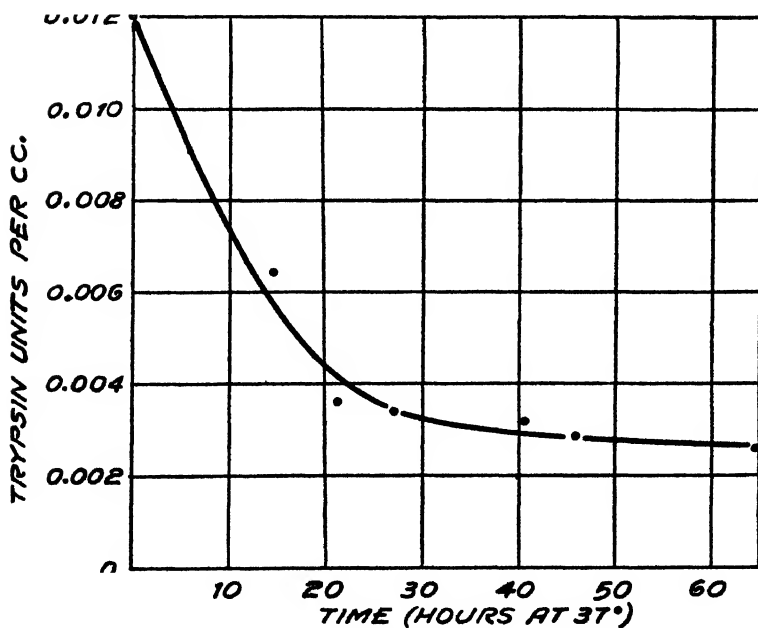


FIG. 2

presence of active trypsin than it is with boiled trypsin, but it would be hazardous to interpret this as due to digestion.

We have determined the activity of the trypsin in our digests, using the method of Anson and Mirsky (9). As is shown in Fig. 2, the tryptic activity decreases upon holding at 37°, but a considerable amount remains after 64 hours.

The effect of trypsin upon urease is not an easy matter to measure since, on the one hand, urease is exceedingly resistant to tryptic digestion and since, on the other hand, urease is rather rapidly inactivated by (as we believe) spontaneous oxidation when kept at 37°. Previous work in this laboratory has indicated that urease is probably a sulfhydryl compound (10), and we have discovered

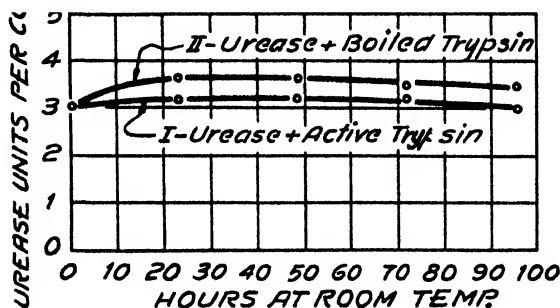


FIG. 3

that an approximately neutral mixture of sulfite and bisulfite will preserve urease solutions effectively.

We have added 3 cc. of urease to a mixture containing 15 cc. of 5 per cent trypsin, 15 cc. of 9.6 per cent neutral phosphate, 15 cc. of water, and 5 cc. of a solution containing 7.8 per cent sodium sulfite and 1.5 per cent sodium bisulfite. Upon being kept at room temperature for 95 hours, there is practically no inactivation of urease either in the presence of active trypsin or boiled trypsin, as is shown in Fig. 3. We have usually observed a moderate increase in urease activity at the start in the solution containing the boiled trypsin and a slight increase in the solution containing the active trypsin. What causes this we cannot say.

Sulfite somewhat hastens the rate of inactivation of trypsin at

room temperature. However, our digests were found to have lost no more than 85 per cent of their tryptic activity after 95 hours.

#### SUMMARY

The conclusion of Tauber and Kleiner that in the presence of gum arabic urease is rapidly inactivated by trypsin is erroneous.

Urease preserved with sulfite was found not to be measurably inactivated by trypsin at room temperature over a period of 95 hours. The sulfite exerted a remarkable preservative action upon the urease.

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## THE METABOLISM OF *d*-XYLULOSE\*

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(Received for publication, December 16, 1936)

*l*-Ketoxylulose was first identified as one of the sugars occurring in pentosuria by Levene and La Forge (1) whose work has been confirmed by numerous other investigators (2). Levene and Tipson (3) have recently proposed the name xylulose for this sugar which exhibits the unusual property of reducing Benedict's qualitative copper reagent in the cold within a few seconds or minutes. It is rarely encountered, in our laboratory, its incidence of occurrence being one in about 50,000. The pentose is usually found in low concentrations, the highest we have observed being less than 1 per cent. Evidence indicates that *l*-ketoxyluluria or *l*-xyluluria is inherited as a recessive character, Lasker, Enklewitz, and Lasker (4) having found thirty-seven cases in twenty families examined. We have undertaken a study of the metabolism of the optical isomer of this interesting sugar. The *l* form or naturally occurring sugar is now being synthesized from *d*-sorbitol, and its metabolism will be reported on at a later date.

Schmidt and Treiber (5) found that when *d*-xylose was refluxed in anhydrous pyridine for 4½ hours, about 14 per cent was converted into *d*-xylulose, along with various other sugars. The isolation of the ketose by means of *p*-bromophenylhydrazine is difficult and the yield poor. Levene and Tipson (3) effected the separation by means of acetoneation of the mixed sugars and subjected the resulting acetone compounds to fractional distillation under a high vacuum. The monoacetone xylulose thus obtained

\* A preliminary report was presented before the Division of Biological Chemistry of the American Chemical Society at Pittsburgh, September, 1936.



yields the pure sugar on hydrolysis. We have adopted their method for the preparation of the ketose.

White rats of the same strain which had been fed a uniform stock diet were used for the experimental work. They were fasted 24 hours and then given 2 cc. of a 25 per cent xylulose solution by stomach tube. After a 3 hour absorption period the animals were anesthetized, sodium amytal being given intraperitoneally; the tissues were frozen *in situ* with CO<sub>2</sub> snow, and then transferred to liquid air. The hind legs were skinned and packed in CO<sub>2</sub> snow. After 4 to 6 minutes the legs were cut off with a pair of scissors and the desired muscles removed from the frozen legs and placed in liquid air. The abdomen was then opened and the liver packed in CO<sub>2</sub> snow. After 4 to 5 minutes the frozen liver was removed and dropped into liquid air. The frozen tissues were then transferred to a tissue-crushing apparatus (6) and reduced to a fine powder by means of a dozen blows of a heavy sledge hammer. The frozen, powdered tissues were transferred to vessels containing the appropriate reagents.

Glycogen, lactic acid, fermentable, and non-fermentable reducing substances were determined in liver and muscle; lactic acid, fermentable, and non-fermentable reducing substances in blood; and fermentable and non-fermentable reducing substances in kidneys, by the procedure and methods described by Blatherwick *et al.* (7, 8).

A 6-fold increase in liver glycogen of females and a 2-fold increase in livers of males above control values show that xylulose is utilized. The difference between values for males and females is undoubtedly due to the fact that the males received much less xylulose per body weight than the females.

In Table I the results for glycogen, lactic acid, fermentable, and non-fermentable reducing substances are given, showing the maximal, minimal, and mean values, together with the mean deviation of the mean. The figures in bold-faced type represent values for rats given xylulose; the others are control values.

No significant change in muscle glycogen or in the lactic acid content of muscle, liver, and blood was observed. Neither was there any significant change in fermentable and non-fermentable reducing substances of muscle, liver, and kidney. There was a tendency toward higher fermentable values for blood.

TABLE I

*Carbohydrate Content of Tissues of Control and Experimental Rats*

The results are expressed in mg. per 100 gm., except for blood in which case they are recorded as mg. per 100 cc. The values for glycogen are given in terms of glucose. Figures in bold-faced type represent values for the rats given *d*-xylulose.

| Substance determined                   | Maxi-<br>mal | Minimal     | Mean        | Mean<br>devia-<br>tion of<br>mean | No. of rats |          |
|--|--------------|-------------|-------------|-----------------------------------|-------------|----------|
|  |              |             |             |                                   | Males       | Females  |
| Glycogen, liver.....                   | 539          | 28          | 221         | 67                                |             | 6        |
| “ “ .....                              | <b>2149</b>  | <b>219</b>  | <b>1275</b> | <b>207</b>                        |             | <b>8</b> |
| “ “ .....                              | 934          | 117         | 502         | 63                                | 15          |          |
| “ “ .....                              | <b>1451</b>  | <b>295</b>  | <b>918</b>  | <b>196</b>                        | <b>5</b>    |          |
| “ muscle.....                          | 672          | 491         | 571         | 28                                |             | 6        |
| “ “ .....                              | <b>679</b>   | <b>576</b>  | <b>619</b>  | <b>13</b>                         |             | <b>8</b> |
| “ “ .....                              | 637          | 434         | 534         | 19                                | 15          |          |
| “ “ .....                              | <b>660</b>   | <b>373</b>  | <b>535</b>  | <b>47</b>                         | <b>5</b>    |          |
| Lactic acid, liver.....                | 17.5         | 7.1         | 11.1        | 0.8                               | 12          | 5        |
| “ “ “ .....                            | <b>21.3</b>  | <b>9.5</b>  | <b>13.9</b> | <b>1.2</b>                        | <b>5</b>    | <b>7</b> |
| “ “ muscle.....                        | 54.2         | 12.7        | 25.7        | 3.0                               | 12          | 5        |
| “ “ “ .....                            | <b>46.3</b>  | <b>12.8</b> | <b>24.7</b> | <b>2.6</b>                        | <b>5</b>    | <b>8</b> |
| “ “ blood.....                         | 10.0         | 9.1         | 9.6         | 0.5                               | 2           | 0        |
| “ “ “ .....                            | <b>18.2</b>  | <b>10.7</b> | <b>13.5</b> | <b>1.1</b>                        | <b>3</b>    | <b>3</b> |
| Fermentable reducing sub-<br>stances   |              |             |             |                                   |             |          |
| Liver.....                             | 146          | 72          | 103         | 5.1                               | 15          | 5        |
| “ .....                                | <b>125</b>   | <b>72</b>   | <b>104</b>  | <b>4.8</b>                        | <b>5</b>    | <b>8</b> |
| Muscle.....                            | 82           | 4           | 21          | 3.9                               | 15          | 5        |
| “ .....                                | <b>30</b>    | <b>9</b>    | <b>18</b>   | <b>2.0</b>                        | <b>5</b>    | <b>8</b> |
| Kidney.....                            | 80           | 25          | 56          | 3.1                               | 15          | 5        |
| “ .....                                | <b>102</b>   | <b>47</b>   | <b>63</b>   | <b>4.4</b>                        | <b>5</b>    | <b>8</b> |
| Blood.....                             | 79           | 57          | 70          | 1.4                               | 4           | 1        |
| “ .....                                | <b>99</b>    | <b>70</b>   | <b>82</b>   | <b>4.4</b>                        | <b>3</b>    | <b>3</b> |
| Non-fermentable reducing<br>substances |              |             |             |                                   |             |          |
| Liver.....                             | 58           | 8           | 31          | 2.8                               | 15          | 5        |
| “ .....                                | <b>57</b>    | <b>19</b>   | <b>35</b>   | <b>3.0</b>                        | <b>5</b>    | <b>8</b> |
| Muscle.....                            | 45           | 10          | 23          | 2.0                               | 15          | 5        |
| “ .....                                | <b>45</b>    | <b>19</b>   | <b>33</b>   | <b>2.5</b>                        | <b>5</b>    | <b>8</b> |
| Kidney.....                            | 37           | 9           | 21          | 1.6                               | 15          | 4        |
| “ .....                                | <b>53</b>    | <b>11</b>   | <b>29</b>   | <b>3.2</b>                        | <b>5</b>    | <b>8</b> |
| Blood.....                             | 9            | 3           | 6           | 0.7                               | 4           | 1        |
| “ .....                                | <b>8</b>     | <b>1</b>    | <b>5</b>    | <b>1.1</b>                        | <b>3</b>    | <b>3</b> |

The non-fermentable reducing substances found in the present series of experiments are much higher than we reported in our xylose metabolism paper (8). This is especially true for muscle. This discrepancy was finally traced to the use of a different sample of Lloyd's reagent. It appears that different preparations of Lloyd's reagent vary greatly in their adsorptive powers.

In a preliminary experiment, eight female rats were given a mixed sugar solution containing approximately 80 per cent xylulose, the remainder consisting of xylose and a small amount of lyxose. The administration of this mixed sugar resulted in a 2-fold increase in liver glycogen content. In previous work (8) we have shown that xylose is not metabolized—no significant change occurring after its administration in either liver or muscle glycogen.

An average increase of 19 mg. in the fermentable sugar of the gastrointestinal tract might lead one to assume that some of the xylulose is converted into a fermentable sugar. However, when xylulose was incubated with an extract of the gastrointestinal tract, we were unable to detect any destruction of the sugar. Xylulose is absorbed from the gastrointestinal tract at an average rate of 131 mg. per 100 gm. of rat per hour. This absorption coefficient is about double that which we found for xylose. Less than 10 per cent of the ingested xylulose appears in the urine.

It was thought that the animal organism might be capable of hydrolyzing monoacetone xylulose and thus utilize the xylulose formed. When the monoacetone compound was administered to rats in amounts equivalent to the xylulose given, there was no evidence of glycogen formation.

In Table II are given glycogen and lactic acid values for liver and muscle after subcutaneous and intraperitoneal injection of xylulose. There appears to be an increase in liver glycogen as compared with the controls, but this is not so marked as when xylulose is ingested. On the other hand muscle glycogen shows a very definite decrease. Why glycogen mobilization should occur from muscle is not known, but this point will receive further investigation as soon as more xylulose is available. Muscle lactic acid values are slightly lower than control values.

As in the case of xylose, a hard translucent mass results in the skin at the site of subcutaneous injection of xylulose. This mass

yields only a trace of non-fermentable sugar, when frozen and analyzed.

The fact that xylulose is more readily metabolized, when ingested, brings up the question as to whether it undergoes some change in the gastrointestinal tract which enables it to be more easily utilized. There is the possibility of phosphoric acid conjugation, although the consensus seems to be that pentoses are not conjugated in the intestine. Inasmuch as xylulose acts more like a hexose than a pentose in its ability to form glycogen, it

TABLE II

*Glycogen and Lactic Acid Content of Liver and Muscle after Injection of Xylulose and Xylose*

The results are expressed in mg. per 100 gm. of tissue. Values for glycogen are given in terms of glucose.

| Rabbit<br>No<br>(females) | Sugar injected         | Muscle   |                | Liver    |                |
|---------------------------|------------------------|----------|----------------|----------|----------------|
|                           |                        | Glycogen | Lactic<br>acid | Glycogen | Lactic<br>acid |
| 1495                      | 500 mg. xylulose*      | 480      | 26 6           | 655      | 12.8           |
| 1770                      | 500 " " *              | 291      | 17 0           | 82       | 10 8           |
| 1775                      | 500 " " *              | 293      | 15 5           | 163      | 13 0           |
| 1773                      | 500 " " †              | 217      | 19 3           | 473      | 10.0           |
| 1774                      | 500 " " †              | 297      | 19.4           | 634      | 9 0            |
| 1796                      | 500 " " †              | 162      | 16 3           | 414      | 9 0            |
| 1794                      | 500 " xylose†          | 527      | 20 4           | 211      | 10 0           |
| 1795                      | 500 " " *              | 504      | 16 4           | 72       | 11 9           |
|                           | Mean of 6 control rats | 571      | 18 9           | 221      | 10.8           |

\* Subcutaneous injection.

† Intraperitoneal injection.

is conceivable that the sugar is metabolized before it is absorbed.

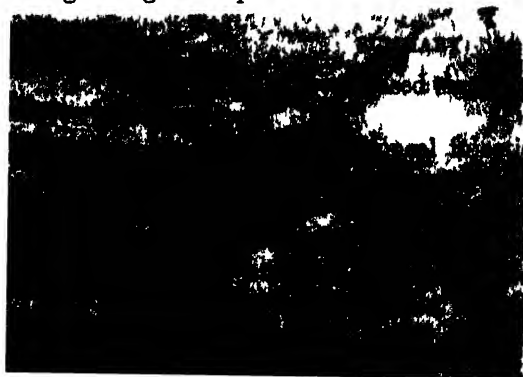
The recent observation that xylulose is easily oxidized at the 5-position suggests that the failure of xylulose to form sugar may be due to its oxidation to a naturally thin sugar.

Whether the origin of the sugar is exogenous or endogenous is

exogenous is exceedingly remote, but the fact must not be overlooked that ascorbic acid and xylulose are closely related. The uniformity of daily excretion of xylulose (10), regardless of diet, seems to preclude any conversion of aldopentose to ketose. Enklewitz and Lasker (10) have recently shown that after the administration of certain drugs, such as pyrimidone and borneol, xylulose excretion is increased in xylulosurics, and that these drugs are excreted as conjugated glucuronates. The increased output of xylulose after ingestion of glucuronic acid convinces them that this acid is a precursor of xylulose. The fact that glucuronic acid may be a precursor suggests that some of it may be formed by enzymatic or bacterial action on normal hexoses. Glucoproteins may also give rise to glucuronic acid. Pentoses, such as *d*-ribose, are necessary components of nucleic acids, and the possibility of some of the xylulose being obtained from these sources must be considered.

The fact that xylulose is a glycogen former shows that this sugar is utilized by the normal animal. We postulate that a normal man is also able to utilize xylulose, but that the pentosuric individual lacks the ability, partially or completely, of using this sugar. The situation would be analogous to that of essential fructosuria. The obvious proof would be to administer xylulose to normal and pentosuric men and to observe the pentose content of the blood and urine. The prohibitive cost of preparing a sufficient amount of xylulose to conduct such an experiment makes the prospect of its performance rather remote.

A study of the reducing values of *d*- and *l*-xylulose with different sugar reagents is planned.



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...kidneys when the sugar

The authors are indebted to Dr. R. S. Tipson for his many helpful suggestions on the preparation of *d*-xylulose and for furnishing nucleating crystals of pure monoacetone xylulose.

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## NON-SUGAR REDUCING SUBSTANCES IN PLANT JUICES\*

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(Received for publication, November 6, 1936)

In a previous publication (1) data were presented which indicated that two commonly used clinical methods for blood sugar gave widely differing values for clarified expressed plant juices.

With pure glucose solutions the methods give results which agree, so that the discrepancies which appeared are probably due to the presence of non-sugar reducing substances in the plant juices. It becomes necessary, therefore, to demonstrate this point so that the results from different methods may be correlated, and also to show the necessity for correcting reducing values given in terms of glucose for the presence of non-glucose reducing substances.

With this in mind recourse was made to the numerous methods, a yeast suspension being used to destroy the fermentable substances previous to the determination of non-fermentable reducing substances (2-6).

### *Methods*

The yeast suspension is prepared by washing 4 gm. of moist yeast cake in a centrifuge until the supernatant liquid is clear. Usually four washings are sufficient. The yeast is then suspended in sufficient water to give a total volume of 40 ml.

To determine the non-fermentable substances, 2 ml. of the yeast suspension are transferred to a 15 ml. centrifuge tube, the suspension is destroyed by centrifuging, and the interior of the tube is thoroughly dried with strips of filter paper. Then 4 ml. of

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either fresh or clarified plant juice are added. The mixture is stirred, placed in a water bath at 37–39°, and frequently agitated. In fresh juices, the acidity varied, averaging about pH 5.50, but in clarified samples (1) the pH was adjusted with 10 per cent disodium phosphate and brom-thymol blue to pH 7.00. Both of these hydrogen ion concentrations are within the optimum range for fresh yeast (7). 20 minutes appeared to be the minimum time necessary to destroy the fermentable fraction, a longer period than that advocated by previous writers.

The sugar methods employed are those of Folin, comparisons being made between his alkaline copper tartrate solution (8) and a modification of his ferricyanide method (9). The modification was introduced to eliminate the use of cyanide (1).

Juice was obtained by the usual method employed in this laboratory. The leaf sample, after the midrib has been removed, is cut or chopped into small pieces. A portion of this sample is then wrapped tightly in cheese-cloth and frozen with carbon dioxide snow as it is formed directly from a coiled copper tube (2 feet long) connected to the carbon dioxide tank. The tissue is completely frozen in 1 to 2 minutes. The cheese-cloth is opened to expose the sample, excess carbon dioxide evaporates, and the tissue thaws. The sample is then placed in a steel cylinder provided with a plunger, and the plant fluids expressed in a hydraulic press. In a properly frozen sample, the juice represents cell solution, some insoluble cell contents, and cellular débris. The insoluble material is removed as completely as possible by centrifuging.

#### EXPERIMENTAL

In a previous paper (1) recovery experiments proved that no important amount of sugar was lost during clarification, as from 96.0 to 101.0 per cent of both added glucose and sucrose was recovered. In this present group of determinations, a number of recovery tests were tried by estimating glucose added to juices freed from fermentable substances by yeast. Of the 2 mg. of glucose added to tomato and celery juices, an average of 1.89 mg., or 94.33 per cent, was recovered.

A number of different plants were used to study the effect of the non-fermentable fraction of plant juice on the determination of

true sugar. Two values are of interest: total reduction and reduction corrected for non-fermentable reducing substances.

TABLE I  
*Reducing Values of Various Fractions of Plant Juices*  
The results are calculated in mg. per ml. of glucose.

| Plant tissues used (leaves)                 | Modified Folin method           |                 |                      | Folin copper method             |                 |                      |
|---|---------------------------------|-----------------|----------------------|---------------------------------|-----------------|----------------------|
|   | Reduction after yeast treatment | Total reduction | True reducing values | Reduction after yeast treatment | Total reduction | True reducing values |
|   | (a)                             | (b)             | (c)                  | (d)                             | (e)             | (f)                  |
| Juices clarified before treating with yeast |                                 |                 |                      |                                 |                 |                      |
| Chrysanthemum.....                          | 1.58                            | 3.07            | 1.49                 | 0.49                            | 2.05            | 1.56                 |
| “.....                                      | 1.50                            | 2.30            | 0.80                 | 0.51                            | 1.22            | 0.71                 |
| Apple.....                                  | 15.54                           | 26.91           | 11.37                | 4.00                            | 15.70           | 11.70                |
| “.....                                      | 6.18                            | 21.73           | 15.55                | 1.08                            | 16.40           | 15.32                |
| Celery.....                                 | 1.24                            | 2.95            | 1.71                 | 0.31                            | 1.87            | 1.56                 |
| “.....                                      | 2.36                            | 9.28            | 6.92                 | 0.55                            | 7.15            | 6.60                 |
| Beet.....                                   | 0.70                            | 2.82            | 2.12                 | 0.16                            | 2.17            | 2.01                 |
| Fresh unclarified juice treated with yeast  |                                 |                 |                      |                                 |                 |                      |
| Tomato.....                                 | 3.45                            | 7.50            | 4.05                 | 0.59                            | 4.97            | 4.38                 |
| “.....                                      | 2.90                            | 8.66            | 5.76                 | 0.69                            | 6.20            | 5.51                 |
| Chrysanthemum.....                          | 1.41                            | 3.40            | 1.99                 | 0.58                            | 2.58            | 2.00                 |
| “.....                                      | 1.08                            | 3.14            | 2.06                 | 0.36                            | 2.00            | 1.64                 |
| Celery.....                                 | 1.45                            | 3.20            | 1.75                 | 0.39                            | 1.88            | 1.49                 |
| Beet.....                                   | 1.66                            | 3.60            | 1.94                 | 0.48                            | 2.55            | 2.07                 |
| Average.....                                | 3.16                            | 7.58            | 4.42                 | 0.78                            | 5.13            | 4.35                 |

Columns *a* and *d*, reduction by 1 ml. of plant juice after treatment with 2 ml. of yeast suspension, at 37–39° for 20 minutes, represent the non-sugar reducing substances; yeast blank subtracted. Columns *b* and *e*, total reduction in 1 ml. of plant juice. Columns *c* and *f*, true sugar values, non-sugar reduction values subtracted from total reduction.

In Table I a comparison of Columns *b* and *e* indicates the large discrepancy to be expected when two differing oxidizing solutions are compared for total reduction. Columns *c* and *f* give the relationship when the methods have been corrected for the non-fermentable fraction. In the first instance, an average of thirteen

determinations by the modified Folin ferricyanide method is 148 per cent of that for the copper solution, whereas after deduction of the non-fermentable blank, the average by the ferricyanide method is only 102 per cent of that for the copper solution, and probably within the experimental error of the two methods. It is evident that in this particular situation the difference between the two methods involved is due to the greater sensitivity of the ferricyanide reagent for the non-sugar reducing substances. In addition to the non-fermentable blank found in plant juices, consideration must be taken of the reducing substances introduced by the yeast. This blank proved to be quite variable, making it necessary to determine its value each time the suspension was used.

TABLE II

*Non-Fermentable Fraction in Alcohol Extracts of Plant Tissues*

The results represent the mg. present in 10 gm. of fresh material, in terms of glucose.

| Plant tissues used (leaves) | Total reducing substances | Non-fermentable substance | Reducing substance as glucose | Non-fermentable substance per 1000 gm. plant tissue |
|-----------------------------|---------------------------|---------------------------|-------------------------------|---|
|                             | mg.                       | mg.                       | mg.                           | gm.   |
| Tomato.....                 | 34.60                     | 17.0                      | 17.60                         | 1.70  |
| Chrysanthemum.....          | 8.80                      | 3.9                       | 4.90                          | 0.39  |
| Bean.....                   | 13.10                     | 4.8                       | 8.30                          | 0.48  |
| Beet.....                   | 16.20                     | 6.0                       | 10.20                         | 0.60  |

It ranged from 0.00 to 0.87 mg. per ml. with the ferricyanide and from 0.00 to 0.16 mg. per ml. with the copper reagent. In all cases the yeast blank gave higher values with the more sensitive ferricyanide reagent. Plant juices, when shaken with permutit or kaolin, show no decrease in the non-fermentable fraction.

In addition to plant juice extracts, determinations were made on four different plants to estimate the non-fermentable fraction present in the alcohol extract of plant material. After the midrib had been removed, the leaf tissues were extracted in the usual manner. The alcohol was completely evaporated and the solutions made up to volume with water. Aliquots of these solutions were cleared by the method used for the clarification of juice

obtained by pressing (1). Total reducing and fermentable sugars were determined.

Table II shows that the non-fermentable fraction varied from a third to a half of the total reduction. In the last column of Table II the non-fermentable fraction is calculated to a basis of 1000 gm. of fresh weight, comparable with the figures presented by Vickery *et al.* (10), which range from 1.89 to 0.68 mg. per 1000 gm. of fresh tobacco leaf.

#### SUMMARY

The differing results given by the two sugar methods used for analysis of expressed plant juice are due to the sensitivity of the oxidizing solutions in reactions with non-fermentable substances. The more sensitive ferricyanide reagent gives higher values for both the non-fermentable fraction and total reduction than does the alkaline copper tartrate solution.

The two methods yield the same values for fermentable sugar, calculated as the difference between reducing values determined before and after yeast treatment.

Alcoholic extracts of tomato, chrysanthemum, bean, and beet likewise show the presence of non-fermentable substances which account for a third to a half of the total reduction under the growth conditions studied.

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## ISOLATION OF CRYSTALLINE TOBACCO MOSAIC VIRUS PROTEIN FROM TOMATO PLANTS\*

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The isolation of a crystalline protein possessing the properties of tobacco mosaic virus has been described (1, 2). This crystalline protein was obtained from the globulin fraction of extracts of diseased Turkish tobacco plants, and was found to be over 100 times as active as the crude juice from the diseased plants used as starting material. The chemical composition, optical rotation, and infectivity of the crystalline protein remained unchanged after ten successive recrystallizations. These facts have suggested that the protein is essentially pure and is the agent responsible for the tobacco mosaic disease.

The tobacco mosaic disease, as is well known, occurs in plants belonging to many different genera of the solanaceous family. The disease was first described in tomato plants by Clinton (3), who showed that the infectious material obtained from tomato plants infected with tobacco mosaic virus produced the same symptom complex on healthy tobacco plants as that caused by infectious material from diseased tobacco plants. As the juice obtained from diseased tomato plants is highly infectious, such plants offered the possibility of providing a new source of material for the isolation of the tobacco mosaic virus protein. If the protein is the infectious agent, then it would necessarily be present in mosaic-diseased tomato plants. The isolation of a similar highly infectious, crystalline protein from diseased tomato plants would prove that this protein is associated with the tobacco mosaic disease in tomato plants and would provide additional evidence

\* A preliminary announcement of this work was published in *Science*, **83**, 85 (1936).

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for the identity of the protein with the infectious agent. The present paper describes the isolation of a crystalline protein from tobacco mosaic-diseased tomato plants and presents the results of comparative studies on the properties of this protein and that isolated from mosaic-diseased tobacco plants.

### EXPERIMENTAL

*Method of Isolation*—The same experimental procedure used in the improved method for the isolation of the crystalline protein from tobacco plants (4) was applied to diseased tomato plants, *Lycopersicon esculentum*, Miller, var. Bonny Best, which had been grown in the field. 2 months after inoculation with the ordinary strain of tobacco mosaic virus (Johnson's tobacco Virus 1), the plants were harvested. They were frozen, ground, and extracted. The first extract from 200 tomato plants gave 105 liters of juice, containing 2.6 mg. of total nitrogen per cc. (by Kjeldahl) and 0.65 mg. of protein nitrogen per cc., while a second extract gave 105 liters of juice containing 1.0 mg. of total nitrogen per cc. and 0.2 mg. of protein nitrogen per cc. The globulin fraction in each extract was precipitated with  $(\text{NH}_4)_2\text{SO}_4$  and filtered. The combined yield of crude globulin amounted to 350 gm. or approximately 0.2 per cent of the weight of the freshly cut plants.

Protein nitrogen was determined by precipitation of the protein with hot 5 per cent trichloroacetic acid. The suspension was immediately cooled and the denatured protein was centrifuged and dissolved in about 1 cc. of 0.2 N NaOH. It was then reprecipitated with about 5 cc. of 10 per cent trichloroacetic acid and again centrifuged. The protein residue was mixed with about 5 cc. of 5 per cent trichloroacetic acid and again centrifuged. It was then transferred to a micro-Kjeldahl flask with 0.2 N NaOH and digested, etc., as for a Kjeldahl analysis.

According to the improved method, the crude globulin was purified by two precipitations with ammonium sulfate, by adsorption on celite at pH 4.5, and by treatment with 0.1 per cent CaO. The application of this procedure to the crude globulin first obtained from mosaic-diseased tomato plants resulted in a yellow pigmented preparation which failed to crystallize. However, it was found that if the procedure described above was repeated several times, the yellow color was gradually lost and the protein

could then be crystallized. The first crystalline material obtained from tomato plants still contained an appreciable amount of yellow color. This could be removed by further fractionation with ammonium sulfate, celite, and CaO.

The application of the improved method to diseased tobacco plants grown under the same conditions in the field as were the tomato plants mentioned above also led finally to white crystalline preparations, but, as in the case of the tomato plants, the crude material had to be fractionated with ammonium sulfate, celite, and CaO more often than when greenhouse plants were used. The amount of treatment necessary to remove the colored impurities from the tobacco protein was, however, considerably less than that required for the tomato protein. Whereas, in the case of the crude tobacco globulin, four to six precipitations with  $(\text{NH}_4)_2\text{SO}_4$  and two or three precipitations on celite were sufficient to give a white opalescent preparation, the crude tomato globulin required approximately twice as much treatment with  $(\text{NH}_4)_2\text{SO}_4$  and celite.

*Measurement of Infectivity*—The relative infectivity of the crystalline virus protein obtained from tomato and tobacco plants, as described above, was determined by the local lesion method of Holmes (5) as modified by Samuel and Bald (6). If infectivity is a characteristic of the crystalline protein, then one would expect it to possess the same infectivity regardless of the plant source. The experiments were, therefore, planned to detect whatever difference, if any, there might be in the infectivity of the different preparations. Samples of virus protein from tobacco plants were compared with samples of virus protein from tomato plants. Solutions to be tested were prepared in dilution series representing concentrations of  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$ , and  $10^{-7}$  gm. of protein per cc. In each test one solution was rubbed on the right halves of all the leaves of half the plants and on the left halves of all the leaves of the remaining half of the plants, while the solution with which it was compared was rubbed on the remaining uninoculated right and left halves of the leaves. Before infectivity measurements were made, the protein solutions were dialyzed until free of salt. Dilutions were then made with 0.1 M phosphate at pH 7. At least twenty, and in some cases from twenty-eight to forty-one, half leaves of *Nicotiana glutinosa*, L., or *Phaseolus vulgaris*, L., var. Early Golden Cluster were used for each concen-



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tration in each test. The relative infectivity of two solutions was then determined by the average number of necrotic lesions produced on the half leaves on which each solution had been rubbed.

In order to evaluate the data obtained, the results of each test at each dilution were treated statistically by "Student's" method (7) on the basis of half leaf units. In this way the mean difference in the number of lesions between the half leaves at one dilution and the standard deviation of the mean difference at that same dilution were obtained. The ratio of the mean difference to the standard deviation was calculated and the odds for significance were determined from Fisher's table of  $t$  (7). The infectivities of two samples were considered significantly different if ratios greater than 2.1, corresponding to odds of about 20:1 or more, were obtained consistently for two or more dilutions in each of two or more tests.

*Relative Infectivity of Virus Protein from Tomato and Tobacco Plants Grown in Field*—The results of a few typical tests on the infectivity of different samples of virus protein from the tomato and tobacco plants described above are shown in Table I. The samples of virus protein from tomato plants, Tomato 1 and 2, as well as those from tobacco plants, Tobacco 1 and 2, were prepared from different batches of crude globulin obtained from plants harvested on different dates. All four samples were purified by the same general procedure. The tomato samples, as mentioned above, required more extensive fractionation than the tobacco samples before they could be crystallized. The preparation, Tomato 3, was obtained from that labeled Tomato 2 by further fractionation with celite and  $(\text{NH}_4)_2\text{SO}_4$ .

The results show that the crystalline virus protein obtained from the tomato plants was definitely less infectious than that isolated from the tobacco plants. The data presented also show that repeated and prolonged fractionation of the virus protein resulted in a gradual loss of activity. The crude globulin obtained from old tomato plants grown in the field contained much dark colored pigment and the virus protein could not be crystallized without the prolonged fractionation mentioned above. Several other attempts to obtain more active preparations from this crude globulin likewise led to crystalline but comparatively inactive products.

*Isolation of Virus Protein from Young Greenhouse Plants*—The results obtained from old tomato plants grown in the field showed that an active crystalline protein similar in properties to that present in mosaic-diseased tobacco plants, but of lower infectivity,

TABLE I  
*Relative Infectivity of Crystalline Virus Protein from Tomato and Tobacco Plants Grown in Field*

| Preparation        | Test plant                 | Concentration<br>(gm. protein per cc.) |                  |                  | Remarks  |
|--------------------|----------------------------|--|------------------|------------------|--|
|                    |                            | 10 <sup>-4</sup>                       | 10 <sup>-5</sup> | 10 <sup>-6</sup> |  |
| Tomato 1           | <i>Nicotiana glutinosa</i> | 68.2*                                  | 6.7              | 2.6              | Tomato 1, slightly yellow crystalline preparation first obtained from plants grown in field. Tobacco 1, sample comparable to Tomato 1 but prepared by less drastic procedure |
| Tobacco 1          |                            | 92.7                                   | 14.3             | 8.45             |  |
| No. of half leaves |                            | 24                                     | 24               | 23               |  |
| M.D./S.D.†         |                            | 2.92                                   | 6.19             | 4.13             |  |
| Tomato 2           | <i>Phaseolus vulgaris</i>  | 69.9                                   | 23.7             | 7                | Tomato 2, second preparation from tomato plants. Tobacco 2, second preparation from tobacco plants   |
| Tobacco 2          |                            | 106.3                                  | 37.3             | 12               |  |
| No. of half leaves |                            | 22                                     | 22               | 20               |  |
| M.D./S.D.          |                            | 4.80                                   | 3.02             | 3.48             |  |
| Tomato 2           | "                          | 83.3                                   | 38.8             | 7.5              | Tomato 3, sample obtained from Tomato 2 after prolonged fractionation, as described in text  |
| " 3‡               |                            | 67.3                                   | 24.3             | 3.7              |  |
| No. of half leaves |                            | 24                                     | 22               | 24               |  |
| M.D./S.D.          |                            | 2.04                                   | 4.42             | 4.15             |  |

\* Numbers opposite a particular preparation represent the average number of necrotic lesions per half leaf obtained on inoculation with the designated preparation and concentration.

† To show a significant difference between the mean number of lesions in any one experiment, the ratio of the difference of the mean (M.D.) to the standard deviation of the difference (S.D.) should not be less than 2.1.

‡ The concentration of Tomato 3 in these tests was 4 times as much as indicated.

could be obtained from mosaic-diseased tomato plants. Because of the difficulties encountered in purifying the protein, however, it could not be determined from the above samples whether the preparations from the two sources represented the same protein, one with a lowered activity due to the more drastic treatment, or

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whether they represented different proteins with different activities. It was also possible that the tomato virus protein differed from the tobacco virus protein, because the tomato plants had been infected for a longer time previous to cutting than the tobacco plants. As shown by Wyckoff, Biscoe, and Stanley (8), there is some variation in the sedimentation constants of the virus proteins obtained from tobacco plants of different ages at different intervals after inoculation. Virus protein obtained from plants 2 weeks after inoculation gave a higher sedimentation constant than that obtained at 4 or 5 weeks after inoculation.

It was desirable, therefore, in comparing the virus protein from diseased tomato and tobacco plants to use plants of approximately the same size, to inoculate them at the same time, and later to harvest the plants after the same period of infection. When young plants grown under greenhouse conditions were used as the source of virus protein, it was found that the purification procedure used for old plants could be simplified and the crystalline protein could be readily obtained from either tobacco or tomato plants. The procedure used in this case was similar to the improved method (4) but differed in several respects. The detailed procedure is described below.

Two flats each of young tobacco and tomato plants, planted twenty-five to a flat, were inoculated when about 3 inches high with 5 cc. of diluted, filtered juice from a mosaic-diseased tobacco plant. The juice contained 0.22 mg. of protein nitrogen per cc. Both the tobacco and tomato plants were cut 4 weeks later, after they had shown symptoms of tobacco mosaic for about 3 weeks. The plants were placed in burlap bags and frozen in a refrigerator at  $-8^{\circ}$ . Each group of plants was ground and extracted as previously described (2). The extracts from the tomato plants after filtration through Hyflo Super-Cel contained a total of 0.82 gm. of protein nitrogen, while those from the tobacco plants contained 1.05 gm. of protein nitrogen. The globulin present in each extract was then precipitated by the addition of 40 gm. of  $(\text{NH}_4)_2\text{SO}_4$  per 100 cc. of extract. The globulin was removed by filtration with the aid of suction on a layer of Hyflo Super-Cel, and the precipitate was washed with about 200 cc. of 40 per cent  $(\text{NH}_4)_2\text{SO}_4$ . The celite was extracted with 1 liter of 1 per cent  $\text{Na}_2\text{HPO}_4$ , U.S.P., and the extract was filtered through a second

layer of celite. The globulin in the filtrate was next precipitated with 20 gm. of  $(\text{NH}_4)_2\text{SO}_4$  per 100 cc. of extract and removed by filtration on Standard Super-Cel (celite). The filter cake was washed with about 100 cc. of 20 per cent  $(\text{NH}_4)_2\text{SO}_4$  and extracted twice with 300 cc. and 200 cc., respectively, of 0.1 M phosphate buffer at pH 7.0. The suspension was filtered each time through a thin layer of celite. The protein in the combined extracts was next precipitated with 15 gm. of  $(\text{NH}_4)_2\text{SO}_4$  per 100 cc. of extract and filtered as before on celite. The filter cake was washed with 15 per cent  $(\text{NH}_4)_2\text{SO}_4$  solution and extracted again with 0.1 M phosphate buffer. The protein was next precipitated with 13 gm. of  $(\text{NH}_4)_2\text{SO}_4$  per 100 cc. and filtered as before on celite, and the celite was washed with a small amount of 13 per cent ammonium sulfate solution. The phosphate extract from the 13 per cent  $(\text{NH}_4)_2\text{SO}_4$  precipitation gave a white opalescent solution of protein with only a trace of yellow color. As the protein was freed from brown pigment, it became less soluble and could be precipitated with a lower concentration of  $(\text{NH}_4)_2\text{SO}_4$ . The use of lower concentrations of ammonium sulfate also aided in the removal of the remaining brown pigment, for the latter was more soluble in the dilute salt solution.

The protein was next precipitated with 20 per cent  $(\text{NH}_4)_2\text{SO}_4$  and was removed by gravity filtration on filter paper. It was scraped from the paper, dissolved in water at pH 7 to 8, and precipitated on an equal weight of celite by adjustment of the hydrogen ion concentration of the solution to pH 4.5. The celite and protein were filtered out on a layer of celite on a Buchner funnel and suspended in 400 cc. of water at pH 7 to 8. The suspension after being thoroughly mixed was filtered, and the virus protein in the filtrate was crystallized by the addition of enough saturated  $(\text{NH}_4)_2\text{SO}_4$  dropwise to cause a slight silkiness in the appearance of the solution, and then by the addition of enough 10 per cent acetic acid in one-half saturated  $(\text{NH}_4)_2\text{SO}_4$  to lower the hydrogen ion concentration to about pH 5.5. Crystallization was completed by the addition of sufficient saturated  $(\text{NH}_4)_2\text{SO}_4$  to bring the salt concentration to approximately 20 per cent by weight. The virus protein from either tomato or tobacco plants may be recrystallized repeatedly without change of activity if the solution is kept cold and the hydrogen ion concentration is

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maintained between pH 5 and 7.5. In fractionation experiments in which virus protein from tobacco plants was recrystallized fifteen times, the final sample proved to have the same infectivity as the original material. The results of the infectivity compari-

TABLE II  
*Relative Infectivity of Virus Protein from Tobacco Plants after One Crystallization and Fifteen Recrystallizations*

| Experi-<br>ment No. | Test No. | Preparation        | Concentration<br>(gm. protein per cc.) |                    |
|---------------------|----------|--------------------|--|--------------------|
|                     |          |                    | $10^{-6}$                              | $5 \times 10^{-6}$ |
| 1*                  | 1        | Crystallized once  | 66.6†                                  | 43.3               |
|                     |          | “ 15 times         | 68.4                                   | 49.0               |
|                     |          | No. of half leaves | 42                                     | 42                 |
|                     | 2        | M.D./S.D.†         | 0.44                                   | 1.4                |
|                     |          | Crystallized once  | 38.6                                   | 34.5               |
|                     |          | “ 15 times         | 35.5                                   | 32.9               |
| 2*                  | 1        | No. of half leaves | 44                                     | 44                 |
|                     |          | M.D./S.D.          | 1.05                                   | 0.47               |
|                     |          | Crystallized once  | 79.8                                   | 38.4               |
|                     | 2        | “ 15 times         | 72.5                                   | 47.0               |
|                     |          | No. of half leaves | 44                                     | 44                 |
|                     |          | M.D./S.D.          | 1.59                                   | 2.57               |
|                     | 2        | Crystallized once  | 51.1                                   | 42.4               |
|                     |          | “ 15 times         | 55.3                                   | 41.9               |
|                     |          | No. of half leaves | 34                                     | 36                 |
|                     |          | M.D./S.D.          | 0.98                                   | 0.12               |

\* In Experiment 1, 30 per cent and in Experiment 2, 81 per cent of the original amount of virus protein was lost in the mother liquor during recrystallization.

† Numbers opposite a particular preparation represent the average number of necrotic lesions per half leaf obtained on *Phaseolus vulgaris* on inoculation with the designated preparation and concentration.

‡ To show a significant difference between the mean number of lesions in any one experiment, the ratio of the difference of the mean (M.D.) to the standard deviation of the difference (S.D.) should be not less than 2.1.

sons for two such samples are shown in Table II. In one experiment 30 per cent and in the other 81 per cent of the original weight of protein was lost during recrystallization. The recrystallizations were carried out by the same procedure used for the original crystallization, with the exception that the solutions were kept at

about 10°. The crystallized protein was removed by centrifugation and the fifteen recrystallizations in each case were completed in 2 days. It may be seen that in both experiments the fifteen times recrystallized sample had the same infectivity as the original, once crystallized material.

The yield of crystalline virus protein obtained from 1.8 kilos of tomato plants by the procedure described above was 1.7 gm., or about 35 per cent of the protein nitrogen present in the extracts. In the case of the tobacco plants, a yield of 3.4 gm. of crystalline virus protein, or approximately 54 per cent of the protein nitrogen



FIG. 1, a

FIG. 1, b

FIG. 1. (a) Tobacco mosaic virus protein from tobacco plants; (b) tobacco mosaic virus protein from tomato plants.  $\times 520$ . (Photographed by J. A. Carlile.)

present in the extracts, was obtained from 1.6 kilos of plant material. Photomicrographs of several times recrystallized samples from these young tomato and tobacco plants are shown in Fig. 1.

*Relative Infectivities of Mosaic Virus Protein from Young Tomato and Tobacco Plants*—The relative infectivities of the crystalline protein obtained from the young tomato and tobacco plants were determined by the method outlined above. The results of four separate tests on *Phaseolus vulgaris* and *Nicotiana glutinosa* are shown in Table III. In one experiment, in which from thirty-seven to forty-one half leaves of *Phaseolus vulgaris* were used for

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TABLE III

Relative Infectivity of Crystalline Virus Protein from Greenhouse Tomato and Tobacco Plants

| Preparation        | Test plant                 | Concentration<br>(gm. protein per cc) |                  |                  |                  | Remarks  |
|--------------------|----------------------------|---------------------------------------|------------------|------------------|------------------|--|
|                    |                            | 10 <sup>-4</sup>                      | 10 <sup>-5</sup> | 10 <sup>-6</sup> | 10 <sup>-7</sup> |  |
| Tomato 5           | <i>Phaseolus vulgaris</i>  | 296 1*                                | 161 7            | 46 1             | 17 6             | Tomato 5 and Tobacco 3, samples from young greenhouse plants                                     |
| Tobacco 3          |                            | 274 8                                 | 146 9            | 36 7             | 14 9             |  |
| No. of half leaves |                            | 37                                    | 40               | 38               | 41               |  |
| M.D./S.D.†         |                            | 1 73                                  | 1 96             | 2 11             | 1 28             |  |
| Tomato 5           | "                          |                                       | 133 0            | 36 3             | 5 4              |  |
| Tobacco 3          |                            |                                       | 123 8            | 38 4             | 3 5              |  |
| No. of half leaves |                            |                                       | 28               | 36               | 41               |  |
| M.D./S.D.          |                            |                                       | 1 01             | 0 48             | 2 62             |  |
| Tomato 5           | <i>Nicotiana glutinosa</i> | 160 0                                 | 95 4             | 43 1             |                  |  |
| Tobacco 3          |                            | 150 6                                 | 103 6            | 41 7             |                  |  |
| No. of half leaves |                            | 23                                    | 30               | 33               |                  |  |
| M.D./S.D.          |                            | 1 95                                  | 1 40             | 0 55             |                  |  |
| Tomato 5           | "                          | 160                                   | 79 8             | 18 7             |                  |  |
| Tobacco 3          |                            | 153                                   | 81 6             | 24 6             |                  |  |
| No. of half leaves |                            | 21                                    | 21               | 24               |                  |  |
| M.D./S.D.          |                            | 1 08                                  | 0 27             | 2 86             |                  |  |
| Tomato 5           | <i>Phaseolus vulgaris</i>  | 194 6                                 | 150 5            | 97 5             | 39 4             | Tobacco 2, same sample from old tobacco plants described in Table I                              |
| Tobacco 2          |                            | 118 0                                 | 81 6             | 35 1             | 10 2             |  |
| No. of half leaves |                            | 19                                    | 24               | 22               | 20               |  |
| M.D./S.D.          |                            | 7 87                                  | 7 55             | 9 55             | 6 02             |  |
| Tomato 6           | "                          | 251 7                                 | 167 7            | 93 9             |                  | Tomato 6, once crystallized sample<br>Tomato 7, same sample after repeated treatment with celite |
| " 7                |                            | 261 7                                 | 150 1            | 53 2             |                  |  |
| No. of half leaves |                            | 35                                    | 37               | 40               |                  |  |
| M.D./S.D.          |                            | 0 85                                  | 2 81             | 7 58             |                  |  |

\* Numbers opposite a particular preparation represent average number of necrotic lesions per half leaf obtained on inoculation with the designated preparation and concentration.

† To show a significant difference between the mean number of lesions in any one experiment, the ratio of the difference of the mean (M.D.) to the standard deviation of the difference (S.D.) should be not less than 2.1.

each of the four dilutions tested, no significant odds for differences in their infectivities were found in three of the four dilutions compared. In another test, in which from twenty-eight to forty-one half leaves of *Phaseolus vulgaris* were used for each of three dilutions tested, only one of the three dilutions gave significant odds for difference. Similar results were found when *Nicotiana glutinosa* was used as the test plant. In two separate tests, in which from twenty-one to thirty-three half leaves of *Nicotiana glutinosa* were used for each of three dilutions tested, only one of the six dilutions gave significant odds for difference. In this and in the other two instances in which differences were found, the odds, however, were not highly significant. As shown in Table III, Tomato 5 and Tobacco 2, the infectivity of the virus preparation from young tomato plants grown in the greenhouse was also compared with that of the protein obtained from old tobacco plants grown in the field. The data show that the virus protein obtained from young plants was significantly more active than that obtained after more prolonged treatment from old plants. In experiments in which the virus protein from young plants was repeatedly treated with celite, it was found that this protein, like that from old plants, became partially inactivated. The infectivity of one such preparation, Tomato 7, which had been recrystallized twelve times and filtered on celite each time, compared to the original sample, Tomato 6, is shown in Table III.

*Relative Infectivity of Juice from Greenhouse Tomato and Tobacco Plants*—The percentage yield of crystalline protein from the young tomato plants was appreciably smaller than that from the young tobacco plants. The yields as mentioned previously were 35 and 54 per cent, respectively, of the total protein nitrogen in the tomato and tobacco extracts. The lower yield in the case of the tomato plants may have been due to a greater loss of the tomato virus protein during fractionation or to its lower relative concentration in the extract. The relative infectivities of extracts from tomato and tobacco plants were, therefore, determined to compare by infectivity measurements the relative concentration of virus present. Both crude freshly expressed juice, obtained by pressing the juice from the ground plants through bandage gauze, and the same after filtration through Hyflo Super-Cel were compared. The extracts from tomato and tobacco plants were analyzed for



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protein and were diluted so that all contained the same protein concentration before they were rubbed on the test plants. The concentrations tested and the method of testing were the same as for the crystalline preparation described above. The results of several tests are shown in Table IV. It may be seen that the crude tobacco extract was significantly more infectious than the

TABLE IV  
*Relative Infectivity of Juice from Greenhouse Tomato and Tobacco Plants*

| Preparation        | Concentration<br>(gm. protein per cc.) |                  |                  | Remarks   |
|--------------------|--|------------------|------------------|---|
|                    | 10 <sup>-4</sup>                       | 10 <sup>-5</sup> | 10 <sup>-6</sup> |   |
| Tomato S.J. 1      | 101.1*                                 | 60.2             | 19.3             | Freshly expressed samples of juice strained through bandage gauze                 |
| Tobacco S.J. 1     | 140.2                                  | 80.3             | 37.3             |   |
| No. of half leaves | 20                                     | 22               | 20               |   |
| M.D./S.D.†         | 4.29                                   | 2.15             | 3.94             |   |
| Tomato S.J. 2      | 191.7                                  | 50.6             | 30.6             | Different from Tomato S.J. 1 and Tobacco S.J. 1, but prepared in same way         |
| Tobacco S.J. 2     | 233.0                                  | 85.3             | 34.3             |   |
| No. of half leaves | 22                                     | 22               | 24               |   |
| M.D./S.D.          | 2.65                                   | 3.85             | 0.97             |   |
| Tomato F.J.        | 148.8                                  | 53.4             | 19.6             | Same as Tomato S.J. 2 and Tobacco S.J. 2 after filtration through Hyflo Super-cel |
| Tobacco F.J.       | 244.6                                  | 88.0             | 37.5             |   |
| No. of half leaves | 22                                     | 22               | 20               |   |
| M.D./S.D.          | 5.68                                   | 4.27             | 4.26             |   |

\* Numbers opposite a particular preparation represent the average number of necrotic lesions per half leaf obtained on *Phaseolus vulgaris* on inoculation with the designated preparation and concentration.

† To show a significant difference between the mean number of lesions in any one experiment, the ratio of the difference of the mean (M.D.) to the standard deviation of the difference (S.D.) should be not less than 2.1.

crude tomato extract on a total protein basis. Since crystalline protein of the same infectivity was obtained from both these extracts, it appears that the difference in infectivity was due to a difference in concentration of the infectious protein. The total protein in the extract of 1.8 kilos of tomato plants was not only less than that in the extract of 1.6 kilos of tobacco plants, but the relative amount of infectious protein was also less. Thus it

appears that the tobacco mosaic virus reaches a higher concentration in tobacco than in tomato plants. The fact that as much as 54 per cent of the total protein in the extract of the tobacco plants was isolated in crystalline form indicates that the major portion of the protein present consists of virus protein.

*General Properties of Crystalline Virus Protein from Tomato Plants*—The crystalline material isolated from mosaic-diseased tomato plants is similar to that obtained from mosaic-diseased tobacco plants, in that it has the general properties of a globulin. It gives a positive test with the usual protein color reactions, such as Millon's, xanthoproteic, glyoxylic acid, and biuret. The

TABLE V  
*Elementary Analysis of Crystalline Tobacco Mosaic Virus Protein from Tomato and Tobacco Plants*

| Preparation* | C               | H               | N (Dumas)       | P               | Ash             |
|--------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|              | <i>per cent</i> | <i>per cent</i> | <i>per cent</i> | <i>per cent</i> | <i>per cent</i> |
| Tomato 5     | 50.93           | 7.58            | 16.70           | 0.21            | 1.29            |
| Tobacco 3    | 50.74           | 7.56            | 16.56           | 0.01            | 0.53            |
| Tomato 2     | 51.57           | 6.91            | 16.20           |                 | 1.64            |
|              | 51.37           | 6.95            | 16.35           |                 | 1.43            |

\* The tomato and tobacco samples are the same as those described in Tables I and III. Tomato 5 and Tobacco 3 were dialyzed against distilled water at pH 7 for 7 days and precipitated and washed with acetone. Tomato 2 was prepared similarly, but was also dialyzed for about 2 days at pH 4.

purified protein is soluble in distilled water at pH 7 and in dilute ammonium sulfate solutions. It is precipitated at pH 7 by concentration of  $(\text{NH}_4)_2\text{SO}_4$  greater than 13 per cent, or by saturation with  $\text{MgSO}_4$ . As the hydrogen ion concentration is increased below pH 7, the protein is precipitated by increasingly smaller amounts of  $(\text{NH}_4)_2\text{SO}_4$ . At pH 3.3 it is practically insoluble even in the absence of salt.

The results obtained from chemical analyses for C, H, N, P, and ash of the highly active crystalline proteins from young tomato and tobacco plants, together with the analysis of one of the less active samples from old tomato plants grown in the field, are shown in Table V. It may be seen that all samples have about the same

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chemical composition. The values for C, H, and N of the highly active tomato sample are in good agreement with those obtained for the tobacco sample of comparable activity.

**Optical Activity**—The results of several comparative determinations of optical activity for preparations of quite different infectivities from both old and young plants are shown in Table VI. They were obtained by determining the rotation of a solution of the purified protein prepared by the addition of 2 drops of 2 N NaOH to 5 cc. of approximately a 0.5 per cent dialyzed suspension of the protein. These likewise show all the samples to have about the same specific rotation, regardless of their infectivity or whether they were prepared from tomato or tobacco plants. It is interesting to note that completely inactive tobacco mosaic virus protein

TABLE VI  
*Optical Activity of Different Samples of Crystalline Tobacco Mosaic Virus Protein*

| Preparation* | $[\alpha]_D^{20}$ (per mg. N) | Concentration          | pH of solution |
|--------------|-------------------------------|------------------------|----------------|
|              | <i>degrees</i>                | <i>gm. per 100 cc.</i> |                |
| Tomato 1     | -0.43                         | 0.57                   | 11.2           |
| " 2          | -0.42                         | 0.88                   | 11.45          |
| " 3          | -0.44                         | 0.46                   | 11.65          |
| " 5          | -0.42                         | 0.49                   |                |
| Tobacco 3    | -0.45                         | 0.40                   |                |

\* The preparations are the same as those described in Tables I and III.

prepared by treatment with ultraviolet light, formaldehyde, or nitrous acid (9) likewise has a specific rotation of  $-0.42^\circ$  per mg. of nitrogen.

**Isoelectric Point**—Cataphoresis experiments were carried out by means of the Northrop-Kunitz apparatus on dilute suspensions of crystals of virus protein. The suspensions were prepared by the addition of 2 drops of saturated  $(\text{NH}_4)_2\text{SO}_4$  solution and 3 to 4 drops of 0.2 N HCl to 10 cc. of a 0.4 per cent solution of the purified protein dialyzed at pH 7 to 8. The suspensions were then diluted with 90 cc. of distilled water adjusted to pH 3.3 with 6 drops of 0.2 N HCl. Portions of this suspension were adjusted to various hydrogen ion concentrations with 0.02 N NaOH and 0.02 N HCl, and were examined in the cataphoresis cell. Under these

conditions, dilute suspensions of tiny crystals easily visible under the microscope were obtained. The hydrogen ion concentration was measured with a MacInnes type glass electrode.

The values found for the isoelectric points of different preparations from both tomato and tobacco plants have varied from about pH 3.2 to about pH 3.35. All the samples examined from either tomato or tobacco plants showed consistent migration towards the negative pole below pH 3.2 and towards the positive pole above pH 3.35. The less active samples obtained from old plants gave isoelectric points in the lower range, while the more active samples obtained from young plants gave values towards the higher range. The most active samples isolated from young tomato and tobacco plants grown in the greenhouse gave the same value of about 3.3. The isoelectric point of an individual preparation was found to vary depending on the salt concentration of the buffer mixture and the nature of the salt present. In experiments in which acetic acid, as well as  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{NaCl}$ , was present, the isoelectric point was raised from 0.1 to 0.3 of a pH unit depending on the concentration of  $(\text{NH}_4)_2\text{SO}_4$  and acetic acid. Eriksson-Quensel and Svedberg (10) have found the isoelectric point of a many times recrystallized sample in  $(\text{NH}_4)_2\text{SO}_4$ -acetate buffer to be pH 3.49. Best (11), from precipitation studies, has reported the isoelectric point of a purified but not crystalline tobacco mosaic preparation to be pH 3.4. Takahashi and Rawlins (12), in experiments in which the migration of virus particles at various hydrogen ion concentrations was determined by infectivity measurements, found the virus to be isoelectric below pH 4.

*Sedimentation Constants*—The sedimentation constants and molecular weights of samples of crystalline tobacco mosaic virus protein from both the old and young tomato plants described above have been determined by Dr. Wyckoff and Mr. Biscoe (8). The sedimentation constant for the crystalline protein isolated from old tomato plants is slightly larger than that found for the protein from tobacco plants. However, the sedimentation constants of the virus proteins prepared under comparable conditions from young tobacco and tomato plants were found to be exactly the same.

Samples of the virus proteins from young tomato and tobacco plants were submitted to Dr. Svedberg for an ultracentrifugal

analysis. During their preparation these samples were maintained between pH 6 and 8 in an effort to retain their molecular homogeneity. The samples, however, arrived too late to be included in the report by Eriksson-Quensel and Svedberg (10). Dr. Svedberg has kindly consented to have the results of the ultracentrifugal study, which was carried out by Mrs. Eriksson-Quensel, presented here. Although the new samples of virus protein were found to be somewhat inhomogeneous with respect to molecular weight, they were more homogeneous than the virus protein previously studied. The sedimentation constant,  $S^{20} \times 10^{13}$ , of virus protein from tobacco plants in solution at pH 6.8 was found to be 197 for the sample submitted in solution and 190 for the sample submitted in the form of a paste. That of virus protein from tomato plants submitted and tested in solution at pH 6.8 was found to be 202. These constants correspond to a molecular weight of about 17,000,000. At pH 9.8 the sample from tomato plants was found to have formed two components having constants of 185 and 125, respectively, and at pH 11.7 it was found to have been disintegrated and split into low molecular weight components having constants of 8.1 and 3.8, respectively. The ultracentrifugal analyses of Dr. Svedberg and Dr. Wyckoff indicate that the sedimentation constant of virus protein obtained from young tomato plants is probably the same as that of virus protein obtained under comparable conditions from young tobacco plants.

*Serological Experiments*—Both precipitation and absorption experiments were performed to compare the serological properties of the crystalline virus protein preparations obtained from tobacco and tomato plants. Antisera were obtained from rabbits which had been injected with 200 mg. of each preparation, in weekly doses of 50 mg. each, dissolved in physiological saline solution at pH 7 to 8. The antiserum from the tobacco virus protein was then tested for precipitating antibodies both with tobacco virus protein and with that obtained from tomato plants, according to the precipitation technique used by Chester (13). The converse experiment was also performed for the antiserum to the tomato virus protein. The results of a typical experiment are shown in Table VII. As previously found (2), the antiserum to tobacco virus protein gave a precipitate with  $2 \times 10^{-4}$  gm. of the homol-

ogous antigen. Likewise, the antiserum to tomato virus protein gave a precipitate with  $2 \times 10^{-6}$  gm. of its homologous antigen. When antisera to either the tobacco virus protein or the tomato virus protein were mixed with the other antigen, a precipitate was likewise formed in each case with but  $2 \times 10^{-6}$  gm. of the antigen.

In the absorption experiments, both antisera were completely absorbed with their heterologous antigens. The absorbed antiserum was then tested with a small amount of its homologous antigen. In neither case was there an additional precipitate. One of two such experiments performed is outlined in Table VIII.

TABLE VII  
*Results of Precipitin Tests with Crystalline Tomato and Tobacco Virus Proteins*

| Concentration of protein used | Antiserum to tomato virus protein | Antiserum to tobacco virus protein | Normal serum |
|-------------------------------|-----------------------------------|------------------------------------|--------------|
| gm.                           |                                   |                                    |              |
| Tomato virus protein          |                                   |                                    |              |
| $2 \times 10^{-4}$            | ++++                              | ++++                               | —            |
| $2 \times 10^{-5}$            | +                                 | +                                  | —            |
| $2 \times 10^{-6}$            | ±                                 | ±                                  | —            |
| Tobacco virus protein         |                                   |                                    |              |
| $2 \times 10^{-4}$            | ++++                              | ++++                               | —            |
| $2 \times 10^{-5}$            | +                                 | +                                  | —            |
| $2 \times 10^{-6}$            | ±                                 | ±                                  | —            |

++++ indicate a very heavy precipitate; + a moderate precipitate; ± a very slight precipitate; — absence of a precipitate.

The results demonstrate that both the virus protein from tobacco and from tomato plants served equally well in absorbing the antibodies formed after the injection of either protein, and that the injection of virus protein from either plant host caused the formation of no antibodies which were not precipitated by the virus protein from the other.

**Solubility Experiments**—The solubilities of the crystalline virus proteins obtained from tomato and tobacco plants were determined to find whether the two samples would give the same solubility. Preliminary experiments, in which the virus proteins obtained from the old tomato and tobacco plants grown in the

TABLE VIII  
*Absorption Experiment on Crystalline Tomato and Tobacco Virus Protein*

| Procedure   | Result | Procedure   | Result |
|---|--------|---|--------|
| (1) 1 cc. antiserum to tomato virus protein + 2.33 cc. (9.3 mg.) tobacco virus protein + 0.3 cc. merthiolate (preservative), * mixed, incubated at 37° for 1 hr., stored in refrigerator overnight, and centrifuged | 20+†   | (1) 1 cc. antiserum to tobacco virus protein + 2.33 cc. (9.3 mg.) tomato virus protein + 0.3 cc. merthiolate (preservative), mixed, incubated at 37° for 1 hr., stored in refrigerator overnight, and centrifuged | 20+†   |
| (2) Supernatant from (1) mixed with 1.0 cc. (3.99 mg.) tobacco virus protein, incubated, etc., as in (1)  | +†     | (2) Supernatant from (1) mixed with 1.0 cc. (3.99 mg.) tobacco virus protein, incubated, etc., as in (1)  | +†     |
| (3) Supernatant from (2) treated as in (2)  | t‡     | (3) Supernatant from (2) treated as in (2)  | t‡     |
| (4) " " (3) " " (2)   | t      | (4) " " (3) " " (2)   | t      |
| (5) " " (4) mixed with 1.0 cc. (3.99 mg.) tomato virus protein, incubated, etc., as in (1)  | t      | (5) " " (4) mixed with 1.0 cc. (3.99 mg.) tobacco virus protein, incubated, etc., as in (1)   | t      |

\* A control experiment, in which normal serum was mixed with the same concentration of merthiolate, failed to give a precipitate.

† The precipitates from (1) and (2) in each experiment were combined, washed twice with physiological salt solution, and analyzed for nitrogen. That from the antiserum to tomato virus protein contained 2.14 mg. of N, while the other contained 2.06 mg. of N. 20+ indicates a large precipitate, + a small precipitate, and t a trace of precipitate.

‡ The trace of precipitate which continued to be formed after the absorption of the antibodies probably consisted of denatured protein.

field were compared, had shown the former to have a solubility about four times that of the latter. However, the tomato virus protein, as was mentioned before, carried more yellow pigment than did the tobacco sample. It was felt, therefore, that the difference might be due to the presence of the yellow impurity in the tomato sample or possibly to its lower infectivity. Subsequent efforts to purify this tomato sample had resulted in preparations that gave white solutions, but, as mentioned before, possessed still less virus activity than the original crystalline material.

Additional solubility determinations were, therefore, carried out on the virus proteins obtained from young tobacco and tomato plants. To 9.0 cc. of dialyzed solutions of the tomato and tobacco virus proteins, each containing 63.8 mg. of protein, was added 1 cc. of 1 M phosphate buffer at pH 5.6 and 625.0 mg. of  $(\text{NH}_4)_2\text{SO}_4$ . Each solution was stirred during the addition of the  $(\text{NH}_4)_2\text{SO}_4$ , and in each case a part of the protein crystallized. Both suspensions were then stirred for  $\frac{1}{2}$  hour and centrifuged for a total of 4 hours. At the end of 2, 3, and 4 hours, respectively, samples were removed from each and analyzed for protein nitrogen.

The supernatant liquid from the tobacco protein contained 2.37 mg. of protein per cc. after centrifugation for 2 hours, while that from the tomato protein contained 1.84 mg. per cc. Additional centrifugation decreased the former value to 2.24 and finally to 1.47 mg. per cc., whereas the tomato decreased to 1.63 and 1.19 mg. per cc. Several experiments of this type in which the amount of ammonium sulfate was varied from 901 mg. to 590 mg. per 10 cc. were performed. In each case it was found that the protein concentration of the supernatant liquid decreased continually during 4 hours of centrifugation, and in all cases the values found for the tobacco protein were slightly higher than those found for the tomato protein. In some cases attempts were made to approach equilibrium from the undersaturated side by extraction of the residue obtained after centrifugation with solvent of the same composition as the solution from which it had separated. In these experiments the protein concentration after 3 hours of centrifugation was always less than the lowest value found when equilibrium was approached from the supersaturated side.

The above experiments provide a rough comparison of the solubilities of the virus protein from tomato and tobacco plants and



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show that they are of the same order. Additional data must be obtained, however, before it can be said with certainty whether the two proteins have identical or slightly different solubilities.

### DISCUSSION

The highly active crystalline protein obtained from young tomato plants by careful fractionation agrees very closely in properties with that isolated from young tobacco plants grown under the same conditions. The general properties of both proteins are the same. They have the same elementary composition, the same optical activity, and the same isoelectric point. The properties of the virus proteins which are most likely to show differences between two preparations are their infectivities, serological reactions, and solubilities. These have been compared for both the tomato and tobacco proteins. A number of infectivity measurements at various concentrations have shown each preparation to have very much the same infectivity. The mean difference between the number of lesions obtained with the same concentration of tomato and tobacco virus protein was always, with but three exceptions, too small to indicate any significant difference in the infectivity of the two samples.

The serological experiments have, likewise, shown the two preparations to have identical serological properties. It is well known that antiserum to one strain of tobacco mosaic virus will give a precipitate when tested either with the same or with a different strain of mosaic virus. However, it has recently been shown by Chester (14) that some strains of tobacco mosaic virus may be differentiated by absorption experiments. It was found that antiserum to ordinary tobacco mosaic virus gave a precipitate with this virus after the serum had been completely absorbed with aucuba mosaic virus. Aucuba mosaic virus, therefore, does not completely absorb the antibodies present after the injection of ordinary tobacco mosaic virus. Absorption experiments on tomato and tobacco virus protein, however, have failed to show differences between these proteins. The antibodies formed after the injection of one protein were completely removed by absorption with the other.

The results of the solubility experiments suggest that the virus protein from tomato plants is slightly less soluble under the same

conditions than virus protein from tobacco plants. However, the differences found were small and could be explained in a number of ways. More experimental data must be obtained before a definite conclusion can be drawn regarding the solubilities of the two proteins. It is entirely possible that the virus protein from tomato plants may differ slightly from that present in tobacco plants but still possess the same physiological properties. As shown by Northrop (15), crystalline pepsins prepared from different animal species possess the same physiological properties but have different solubilities and are therefore different proteins. It seems possible that slightly different proteins might also carry the same virus activity.

The isolation of crystalline protein preparations with varying degrees of infectivity from old tomato plants has shown that the loss of activity does not affect the ability of the protein to crystallize. This result is comparable to what has been found for aucuba mosaic virus (16) and even more strikingly for tobacco mosaic virus treated with hydrogen peroxide or nitrous acid (9). Aucuba mosaic virus protein, like tobacco mosaic virus protein, becomes less active on repeated fractionation, while tobacco mosaic virus protein becomes entirely inactive when treated with hydrogen peroxide or nitrous acid. Both the less active aucuba and the entirely inactive hydrogen peroxide-treated proteins are well defined crystalline preparations. The fact that the changed protein obtained after hydrogen peroxide treatment resembles very closely the partially inactive samples suggests that the partial inactivation may be due to a similar type of reaction. Repeated treatment with celite as used in the course of fractionation may well catalyze the oxidation by air of unstable groups in the molecule.

#### SUMMARY

The isolation from tobacco mosaic-diseased tomato plants of a crystalline protein possessing the properties of tobacco mosaic virus is described. It was found that the most active crystalline material could be best obtained from young rapidly growing greenhouse plants by a procedure involving a minimum amount of treatment with celite.

The properties of the proteins obtained from tomato and tobacco

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plants grown under the same conditions and treated by the same procedure have been compared. The proteins have been shown to possess the same infectivities and to have identical serological properties and very nearly the same solubilities. They likewise have the same chemical composition, optical activity, and isoelectric point, and give the same sedimentation constant.

Repeated fractionation of the virus protein with celite at pH 4.5 and 8.0 results in a gradual inactivation of the protein, which remains soluble and may still be crystallized. It has been shown in other fractionation experiments, however, in which as much as 81 per cent of the original sample has been lost during the course of fifteen recrystallizations, that the crystals which remained possessed the same infectivity as the original sample.

The comparison of the relative infectivities of the juices of diseased tomato and tobacco plants on a total protein basis indicates, in agreement with the percentage yields of crystalline virus protein isolated, that the tobacco mosaic virus reaches a higher concentration in tobacco than in tomato plants.

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## **CHEMICAL STUDIES ON THE VIRUS OF TOBACCO MOSAIC**

### **IX. CORRELATION OF VIRUS ACTIVITY AND PROTEIN ON CENTRIFUGATION OF PROTEIN FROM SOLUTION UNDER VARIOUS CONDITIONS**

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The question as to whether the crystalline virus protein isolated from mosaic-diseased Turkish tobacco plants (1) is tobacco mosaic virus or is a mixture or combination of virus and inactive material is one of importance. The problem does not lend itself to direct positive experimental proof, and it appears that here, as with the enzymes (2), the proof of identity or of non-identity of protein and biological activity must consist largely of indirect evidence. Although considerable evidence that the virus activity is a specific property of the protein has been accumulated (1, 3-10), it has seemed desirable to continue attempts to separate the virus activity from the protein.

The unusually high molecular weights of the tobacco mosaic virus proteins, which are of the order of 17,000,000 and are considerably larger than the molecular weight of any other known protein, make it possible to centrifuge their molecules from solution by means of a high speed centrifuge (7, 8). This property permits a new and powerful attack to be made on the problem as to the identity of virus activity and protein, for it enables the protein to be removed selectively from solution by centrifugal force under a variety of conditions. If the virus activity is a specific property of the high molecular weight protein, then, following centrifugation of different amounts of protein from solutions at different hydrogen ion concentrations or from solutions containing other proteins, the virus activity of the supernatant liquids should be diminished and be proportional to the amount

of high molecular weight virus protein that they contain. If, on the other hand, the virus activity should be due to a separate entity adsorbed on the protein or to a dissociable group attached to the protein, it seems probable that the virus activity of the supernatant liquids would not always be proportional to their high molecular weight protein content. It would be expected that separation of virus activity from high molecular weight protein through reversing the charge on the protein, or by adding other proteins, or by dissociation of active group and protein at certain hydrogen ion concentrations might occur, and then, following centrifugation, the virus activity of the supernatant liquids would be much greater than that which would be proportional to the high molecular weight protein content. These possibilities were tested experimentally by centrifuging the high molecular weight protein at the isoelectric point, on either side of the isoelectric point, and also from solutions containing low molecular weight proteins such as tobacco proteins, egg albumin, trypsin, and pepsin. The results of these experiments are presented in this paper.

#### EXPERIMENTAL

*Centrifugation of Virus Protein from Solutions Containing Low Molecular Weight Proteins*—It was found that the high molecular weight virus protein could be centrifuged from solution from the juice of mosaic-diseased Turkish tobacco plants to form a crystalline mass at the bottom of the tube by means of an ultracentrifuge operating at a speed of about 25,000 R.P.M. for about 3 hours (8, 9). The supernatant liquid had the same appearance as the juice had before centrifugation, despite the fact that about 4 mg. of protein per cc. had been removed from the solution. The supernatant liquid was found to contain considerable pigment and uncentrifugable, low molecular weight protein. If the virus is simply adsorbed on a "carrier" protein, it seemed possible that the activity might be divided between the low molecular weight protein in the supernatant and the high molecular weight protein in the sedimented mass. The virus activity of the protein in the upper portion of the supernatant liquid, of the protein in the middle portion of the supernatant liquid, and of the protein in the crystalline mass at the bottom of the tube was determined.

The results, which are given in Table I, show that the low molecular weight protein in the upper and middle portions of the supernatant liquid possessed practically no virus activity, and that the mass of high molecular weight protein at the bottom of the tube possessed practically all of the virus activity. Similar results were obtained when solutions containing about 5 mg. of virus protein per cc. and from 1 to 10 mg. of egg albumin, trypsin, and pepsin per cc. respectively in 0.1 M phosphate buffer at hydrogen ion concentrations between pH 6 and 9, were subjected to a short run in an air-driven ultracentrifuge equipped with a quantity head (11). In these experiments, therefore, no indication

TABLE I  
*Activities of Upper and Middle Portions of Supernatant Liquid and of Sedimented Protein Following Ultracentrifugation of Juice from Diseased Plants*

The solution of sedimented protein was prepared by dissolving the crystalline mass of protein at the bottom of the centrifuge tube in a volume of 0.1 M phosphate buffer at pH 7 equal to the volume of the juice sample used for sedimentation. All dilutions were made with 0.1 M phosphate buffer at pH 7.

| Dilution                         | Undiluted | 1:10  | 1:100 | 1:1000 | 1:10,000 | 1:100,000 |
|----------------------------------|-----------|-------|-------|--------|----------|-----------|
| Upper portion, supernatant...    | 0.1*      | 0.2   | 0.0   |        |          |           |
| Middle " " ....                  | 0.5       | 0.3   | 0.1   | 0.0    | 0.0      |           |
| Solution of sedimented protein.. | 417.2     | 488.7 | 224.3 | 135.5  | 31.2     | 5.8       |

\* The numbers represent the average number of lesions per leaf obtained on ten leaves of *Nicotiana glutinosa* on inoculation with the designated preparation and dilution.

that an active group could be separated from the high molecular weight protein and attached to low molecular weight proteins was obtained. In solution and in the presence of other proteins, therefore, the virus activity remained closely associated with the high molecular weight protein.

*Correlation of Virus Activity and Protein on Centrifugation of Neutral Protein or of Negatively or Positively Charged Protein Ions*—Aucuba mosaic, a strain of tobacco mosaic virus, was used in one experiment because it possesses a higher molecular weight and a more alkaline isoelectric point than does tobacco mosaic protein (4, 8). These properties enable experimentation at more acid

hydrogen ion concentrations with respect to isoelectric point and more rapid sedimentation than would be possible with ordinary tobacco mosaic protein. The isoelectric point of aucuba mosaic virus protein is pH 3.7, at which hydrogen ion concentration the protein may be regarded as neutral or uncharged. At higher hydrogen ion concentrations the protein forms positively charged ions and at lower hydrogen ion concentrations it forms negatively charged ions. If the virus activity is a specific property of the high molecular weight protein, then, following centrifugation of most of the neutral protein or of either negatively or positively charged protein ions from solution, the activity of the supernatant liquid should be greatly reduced and be proportional to the amount of high molecular weight protein that it contains. If, on the other hand, the virus activity should be due to an entity adsorbed on inactive high molecular weight protein, it should be released under certain conditions. If such virus agent is large, its release from the protein should cause the sedimentation constant of the protein to be changed. Experimentally, however, the sedimentation constant of the aucuba mosaic protein was found to be essentially unchanged over the range from pH 2.2 to 9.3 (8). Therefore, if a virus agent is released at certain hydrogen ion concentrations, such agent must be very small compared to the protein; hence centrifugation of the high molecular weight protein from solution should separate it from the virus activity and result in a supernatant liquid possessing an activity much greater than that which would be proportional to the protein that it contains. Such an entity might possess an isoelectric point or possess a constant positive or negative charge. However, since the tobacco mosaic virus proteins are negatively charged over the pH range where they are usually worked with, it appears more probable that an adsorbed entity would carry a positive charge. It is possible that it might carry a constant positive charge or that it might be isoelectric at a low hydrogen ion concentration and hence be positively charged only over the range where the virus protein is negatively charged. If such an entity should carry a constant positive charge, it should be released and hence separable by centrifugation of the protein at hydrogen ion concentrations greater than pH 3.7. If the entity should be isoelectric at a low hydrogen ion concentration, it would possess a positive charge

and hence should also be separable at hydrogen ion concentrations greater than pH 3.7. Should the entity possess a constant negative charge or be isoelectric at a hydrogen ion concentration greater than those used for centrifugation, it should be separable at hydrogen ion concentrations less than pH 3.7. If the entity should possess an isoelectric point between the pH values used for centrifugation, it would possess the same charge as that of the protein and hence should be separable at both pH values. If the activity should not be due to a separate entity but to a dissociable group, similar to the heme of hemoglobin, then at certain hydrogen ion concentrations it should be possible to cause dissociation of such a group. It seems likely that such dissociation would occur at a very acid or very alkaline pH. Unfortunately the virus proteins become denatured and inactive at hydrogen ion concentrations greater than about pH 2 or less than about pH 9 (12); hence it was not possible to work at more acid or alkaline reactions. The possibilities discussed above were explored by means of experiments in which aucuba mosaic virus protein was centrifuged as neutral or uncharged protein at pH 3.7, as positively charged ions at pH 2.4, and as negatively charged ions at pH 7.3, and in which tobacco mosaic virus protein was centrifuged as positively charged ions at pH 2.4, and as negatively charged ions at pH 6.7 and 9.4.

There is considerable error involved in the measurement of the activity of a virus preparation; hence, in order to correlate activity with protein, it was necessary to adjust the conditions so that the reduction in the protein concentration of the supernatant liquid on centrifugation would be large compared to this error. It had been found in previous experiments that centrifugation of a solution containing about 20 mg. of virus protein per cc. for about 7 hours on a Swedish angle centrifuge at about 5000 R.P.M. would result in the sedimentation of about 95 per cent of the protein from an easily recognizable upper layer of the supernatant liquid. In these experiments a small mass of protein sedimented to the bottom of the tube and the solution above this mass was quite opalescent, except for the uppermost 20 per cent which formed a definite layer of clear solution. This clear layer was usually found to contain about 1 mg. of protein per cc. The reduction of the protein concentration from about 20 mg. per cc. to about 1 mg. per



cc. represents a difference which is large compared to the errors in activity measurements, and thus makes possible a significant evaluation of the relative activities of the two solutions. At the same time, there is sufficient protein remaining in the supernatant liquid so that the exact amount may be estimated chemically with considerable accuracy. It is obvious that insufficient sedimentation might result in a solution having an activity which might not be significantly different from that of the original solution, and that too complete centrifugation might result in a solution containing an amount of protein too small to be estimated accurately by chemical analysis. In the experiments being reported, therefore, the conditions previously mentioned were adopted in order to secure the desired amount of sedimentation.

Aucuba mosaic virus protein which had been prepared 3 months previously by means of the improved method (13), in which the protein was subjected to hydrogen ion concentrations between pH 4.5 and 8, was used in the first experiment. 60 cc. of a solution containing 13.8 mg. of the protein per cc. were adjusted to pH 3.7 and centrifuged for 15 minutes at about 3000 R.P.M. The virus proteins are insoluble at their isoelectric points and may be sedimented by means of an ordinary laboratory centrifuge. The supernatant liquid was found to contain no demonstrable protein and to possess no virus activity. This demonstrates that the activity remains closely associated with the protein when it is neutral or uncharged. The clear supernatant liquid was discarded and the white precipitate was suspended in 30 cc. of water. The protein was taken into solution by adjusting the suspension to pH 7 and the solution was then dialyzed against water at pH 8 for 4 hours. The 35 cc. of protein solution which was at pH 7.3 were found to contain 23.0 mg. of protein per cc. following centrifugation for 10 minutes to remove a small amount of denatured protein. A 16 cc. portion was adjusted to pH 2.4 by the careful, gradual addition of 0.9 cc. of 0.2 N HCl and, following centrifugation for 10 minutes, was found to contain 21.8 mg. of protein per cc. As the hydrogen ion concentration of the 16 cc. of fairly clear, opalescent protein solution was increased, the solution first took on the characteristic silky, shimmering appearance, then near the isoelectric point it became densely white and quite viscous, and finally, near pH 2.5, it became fairly

clear again. When first prepared, there was no noticeable difference in the appearance of the solutions of protein at pH 7.3 and 2.4. On standing, however, the solution at pH 2.4 became turbid, probably due to the formation of denatured protein.

Immediately following the adjustment of the one sample to pH 2.4, 15 cc. samples of protein solution at pH 7.3 and 2.4 were placed in a Swedish angle centrifuge in a room held at about 5° and spun for 7 hours at about 5000 R.P.M. Uncentrifuged samples of the solutions at pH 7.3 and at pH 2.4 were also kept in the cold room during this time. The centrifuged and uncentrifuged samples were removed from the cold room and the upper clear layers, consisting of about 3 cc. in the case of the solution at pH 7.3 and of about 4 cc. in the case of the solution at pH 2.4, were pipetted from the tops of the centrifuged solutions. To these two supernatant liquids and the two uncentrifuged samples was then immediately added sufficient 2 M phosphate buffer at pH 7 to adjust the phosphate concentration to 0.1 M. This served to bring the solutions formerly at pH 2.4 to about pH 6. The supernatant liquid of the solution centrifuged at pH 2.4 was found to contain 1.1 mg. of protein per cc. and that of the solution centrifuged at pH 7.3 was found to contain 1.3 mg. of protein per cc.; hence in each case about 95 per cent of the protein had been sedimented and removed from the supernatant liquid. The virus activity of the supernatant liquids was then compared with that of the uncentrifuged samples by means of the half leaf method of inoculation on plants of *Nicotiana glutinosa*, L., and *Phaseolus vulgaris*, L., var. Early Golden Cluster (14). As may be seen from Table II, the dilutions of the uncentrifuged samples were much more active than the corresponding dilutions of the supernatant liquids of the centrifuged samples. In the case of the undiluted samples of the preparations at pH 7.3, more lesions were obtained with the supernatant liquid than with the original solution containing about 18 times more protein. This anomalous result in which a concentrated virus protein solution causes fewer lesions than certain of its dilutions has been observed occasionally, especially in the case of solutions containing about 10 or more mg. of protein per cc. This phenomenon rarely occurred when solutions containing less than about 1 mg. per cc. of virus protein were used. Results obtained with solutions containing from  $10^{-3}$  to  $10^{-4}$  gm. of pro-

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tein per cc., and preferably  $10^{-4}$  or  $10^{-5}$  gm. per cc., are considered most significant. In the present experiment it was not possible to repeat the test with this particular solution because of the small amount of solution available. However, the fact that three out of four tests of the preparations originally at pH 7.3 and that all tests of the preparations originally at pH 2.4 resulted in the uncentrifuged samples producing many more lesions than the cor-

TABLE II

*Comparison of Virus Activity of Solutions of Aucuba Mosaic Protein before and Following Centrifugation on Angle Centrifuge at pH 7.3 and 2.4*

Tests in which uncentrifuged samples of protein and centrifuged samples of protein were diluted without regard to protein content. All dilutions were made with 0.1 M phosphate buffer at pH 7.

| pH during centrifugation | Protein per cc. before dilution | Sample                              | Dilution of samples |      |       |        |
|--------------------------|---------------------------------|-------------------------------------|---------------------|------|-------|--------|
|                          |                                 |                                     | Undiluted           | 1:10 | 1:100 | 1:1000 |
| 7.3                      | mg.<br>23.0                     | Uncentrifuged                       | 131.3*              | 86.5 | 69.0  | 9.8    |
|                          | 1.3                             | Supernatant from centrifuged sample | 156.5†              | 22.5 | 7.6   | 1.2    |
| 2.4                      | 21.8                            | Uncentrifuged                       | 159.0               | 84.3 | 44.7  | 22.5   |
|                          | 1.1                             | Supernatant from centrifuged sample | 106.0               | 24.6 | 8.8   | 1.7    |

\* The numbers represent the average number of lesions per half leaf obtained on eighteen or more half leaves of *Phaseolus vulgaris* on inoculation with the designated preparation and dilution.

† The numbers represent the average number of lesions per half leaf obtained on the other halves of the same leaves on inoculation with the designated preparation and dilution. The halves of the leaves to which a given sample was applied were reversed at each dilution.

responding supernatant liquids is regarded as demonstrating that the loss of about 95 per cent of the protein by centrifugation is accompanied by a loss of virus activity.

In order to determine whether or not the decrease in virus activity was proportional to the decrease in protein concentration, dilutions of the uncentrifuged samples in 0.1 M phosphate buffer at pH 7 were made up containing  $10^{-2}$ ,  $10^{-4}$ ,  $10^{-5}$ , and  $10^{-6}$  gm.

of protein per cc., respectively, and then tested against dilutions of the corresponding supernatant liquids of the centrifuged samples

TABLE III

*Correlation of Virus Activity and Protein on Centrifugation of Aucuba Mosaic Protein at pH 7.3 and 2.4*

Tests following dilution of uncentrifuged samples to the same protein concentration as in the corresponding supernatant liquids of centrifuged samples. All dilutions were made with 0.1 M phosphate buffer at pH 7.

| pH during centrifugation | Test plant                 | Preparation                         | Protein concentration used for tests, gm. per cc. |                  |                  |                  |
|--------------------------|----------------------------|-------------------------------------|---|------------------|------------------|------------------|
|                          |                            |                                     | 10 <sup>-2</sup>                                  | 10 <sup>-3</sup> | 10 <sup>-4</sup> | 10 <sup>-5</sup> |
| 7.3                      | <i>Phaseolus vulgaris</i>  | Uncentrifuged                       | 122.1*  | 45.4             | 26.6             | 6.8              |
|                          |                            | Supernatant from centrifuged sample | 166.8   | 49.7             | 35.5             | 8.0              |
|                          |                            | No. of half leaves                  | 28  | 24               | 26               | 28               |
|                          | <i>Nicotiana glutinosa</i> | M.D./S.D.†                          | 4.85  | 1.35             | 3.12             | 1.52             |
|                          |                            | Uncentrifuged                       | 67.3  |                  |                  |                  |
|                          |                            | Supernatant from centrifuged sample | 68.8  |                  |                  |                  |
| 2.4                      | <i>Phaseolus vulgaris</i>  | No. of half leaves                  | 8   |                  |                  |                  |
|                          |                            | M.D./S.D.                           | 0.39  |                  |                  |                  |
|                          |                            | Uncentrifuged                       | 111.0   | 60.2             | 20.7             | 4.0              |
|                          |                            | Supernatant from centrifuged sample | 117.8   | 64.5             | 16.5             | 4.7              |
|                          | <i>Nicotiana glutinosa</i> | No. of half leaves                  | 26  | 24               | 26               | 24               |
|                          |                            | M.D./S.D.                           | 0.46  | 0.66             | 1.82             | 1.44             |
|                          |                            | Uncentrifuged                       | 49.4  |                  |                  |                  |
|                          |                            | Supernatant from centrifuged sample | 43.5  |                  |                  |                  |
|                          |                            | No. of half leaves                  | 8   |                  |                  |                  |
|                          |                            | M.D./S.D.                           | 0.96  |                  |                  |                  |

\* The numbers opposite a given preparation represent the average number of lesions per half leaf obtained on inoculation with the designated preparation and concentration. A given preparation was administered to the right halves of half of the leaves and to the left halves of the remaining leaves in each test.

† To show a significant difference between the mean number of lesions in any one experiment, the ratio of the difference of the mean (M.D.) to the standard deviation of the difference (S.D.) should not be less than 2.1.

containing similar amounts of protein. The results, which were subjected to a statistical study (6), are presented in Table III and show that in eight of the ten tests there was no significant differ-

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ence in the number of lesions produced by the supernatant liquids and by the corresponding uncentrifuged samples when tested at the same protein concentration. This is an indication that the activity of the supernatant liquids was proportional to the amount of protein that they contained, and hence that, as the negatively or positively charged protein ions were centrifuged from solution, the decrease in virus activity was proportional to the decrease in the concentration of the high molecular weight protein.

The possibilities discussed at the beginning of this section were also tested experimentally with tobacco mosaic virus protein at three different hydrogen ion concentrations. Since this protein has a lower sedimentation constant than has aucuba mosaic protein (8), it was necessary to centrifuge it for a longer period of time. It was also found preferable to compare the activities of the upper portions with the respective lower portions of the centrifuged samples instead of with uncentrifuged samples. In this experiment, three portions of a solution containing 12 mg. of tobacco mosaic virus protein per cc., freshly prepared from young greenhouse plants and maintained at pH 7 during the preparation, were adjusted to pH 2.4, 6.7, and 9.4, respectively, and centrifuged for 10 hours on a Swedish angle centrifuge in the cold room. At the end of this time the upper and lower portions of the solution at pH 2.4 contained 1.2 and 28.3 mg. of protein per cc., respectively, those of the solution at pH 6.7 contained 1.2 and 16.8 mg. of protein per cc., respectively, and those of the solution at pH 9.4 contained 3.0 and 17.6 mg. of protein per cc., respectively. The protein concentration in the lower portion was about 24 times that in the upper portion in the case of the solution at pH 2.4, 14 times in the case of the solution at pH 6.7, and about 6 times in the case of the solution at pH 9.4. Immediately after their separation, these different fractions were adjusted to pH  $6.5 \pm 0.5$ . The bottom portions were diluted with 0.1 M phosphate buffer at pH 6.8 so that they contained the same protein concentration as their respective upper portions and were again analyzed for protein content. The activities of the respective upper and diluted lower portions were then compared at dilutions of  $10^{-4}$  and  $10^{-8}$  gm. of protein per cc., by the half leaf method. It may be seen from Table IV that in six of the eight tests there was no significant difference between the activities of the upper portions of the solu-

TABLE IV

*Correlation of Virus Activity and Protein on Centrifugation of Tobacco Mosaic Protein at pH 2.4, 6.7, and 9.4*

Tests following the dilution of the lower portions to the same protein concentration as in the corresponding upper portions of centrifuged samples. All dilutions were made with 0.1 M phosphate buffer at pH 7. *Phaseolus vulgaris* was used as the test plant.

| pH during centrifugation | Test No. | Protein concentration after centrifugation   | Portion of centrifuged sample used | Protein concentration used for tests, gm. per cc. |                  |
|--------------------------|----------|--|------------------------------------|---|------------------|
|                          |          |  |                                    | 10 <sup>-4</sup>                                  | 10 <sup>-5</sup> |
| 2.4                      | 1        | mg. per cc.<br>1.2                           | Upper                              | 57.9*   | 25.3             |
|                          |          | 28.3   | Lower                              | 62.1  | 30.4             |
|                          |          |  | No. of half leaves                 | 52  | 52               |
|                          |          |  | M.D./S.D.†                         | 0.96  | 2.34             |
|                          | 2        | 1.2  | Upper                              | 58.1  | 30.6             |
|                          |          | 28.3   | Lower                              | 68.3  | 30.9             |
|                          |          |  | No. of half leaves                 | 56  | 56               |
|                          |          |  | M.D./S.D.                          | 2.51  | 0.15             |
| 6.7                      | 1        | 1.2  | Upper                              | 145.0   | 74.8             |
|                          |          | 16.8   | Lower                              | 161.2   | 82.8             |
|                          |          |  | No. of half leaves                 | 56  | 56               |
|                          |          |  | M.D./S.D.                          | 2.02  | 1.89             |
|                          | 2        | 1.2  | Upper                              | 70.3  | 22.0             |
|                          |          | 16.8   | Lower                              | 79.9  | 25.8             |
|                          |          |  | No. of half leaves                 | 52  | 52               |
|                          |          |  | M.D./S.D.                          | 2.02  | 2.03             |
| 9.4                      | 1        | 3.0  | Upper                              | 81.0  | 40.2             |
|                          |          | 17.6   | Lower                              | 111.0   | 58.0             |
|                          |          | (Not corrected for uncentrifugable material) | No. of half leaves                 | 56  | 56               |
|                          |          |  | M.D./S.D.                          | 6.38  | 5.49             |
|                          | 2        | 2.1  | Upper                              | 128.6   | 67.6             |
|                          |          | 16.7   | Lower                              | 142.1   | 74.5             |
|                          |          | (Corrected for uncentrifugable material)     | No. of half leaves                 | 56  | 56               |
|                          |          |  | M.D./S.D.                          | 2.09  | 1.91             |

\* The numbers opposite a given preparation represent the average number of lesions per half leaf obtained on inoculation with the designated preparation and concentration. A given preparation was administered to the right halves of half of the leaves and to the left halves of the remaining leaves in each test.

† To show a significant difference between the mean number of lesions in any one experiment, the ratio of the difference of the mean (M.D.) to the standard deviation of the difference (S.D.) should not be less than 2.1.

tions at pH 2.4 and 6.7 and their respective lower portions when tested at the same protein concentration and that in the other two tests the difference was not highly significant. This shows that the activity of each portion was proportional to the protein that it contained.

In the case of the solution at pH 9.4 the results indicated that the upper portion was significantly less active per mg. of protein than the lower portion. It is known that inactivation of tobacco mosaic virus occurs as the hydrogen ion concentration is lowered beyond about pH 9 (12), and that at pH 9.8 tobacco mosaic virus protein breaks down into lower molecular weight components (6). It appeared likely, therefore, that the upper portion at pH 9.4 might be less active due to the presence of inactive, low molecular weight, hydrolytic products of the virus protein. The fact that the protein concentration in the upper portion of the preparation centrifuged at pH 9.4 was over twice that of the upper portions of the preparations centrifuged at pH 6.7 and 2.4 is a further indication of the presence of uncentrifugable material in the preparation at pH 9.4. In order to determine the amount of uncentrifugable material present, a portion of the preparation previously centrifuged at pH 9.4 was spun on an air-driven ultracentrifuge under conditions which, as will be shown in a later paragraph, result in the complete sedimentation of the high molecular weight virus protein. Samples of the preparations formerly at pH 6.7 and 2.4 were also centrifuged at the same time. Following ultracentrifugation the supernatant liquid of the preparation formerly at pH 9.4 was found to contain 0.9 mg. of inactive, uncentrifugable protein per cc., whereas the other two supernatant liquids contained only approximately 0.1 mg. of uncentrifugable material per cc. Since the original solution contained 12 mg. of protein per cc., of which approximately 0.1 mg. per cc. was uncentrifugable, about 7 per cent of the virus protein was converted into inactive, uncentrifugable material during the time the solution was kept at pH 9.4. It would be quite difficult to detect this amount by activity measurements, but when the relative amount of inactive, uncentrifugable material was increased to 30 per cent through removal of high molecular weight virus protein by sedimentation, it became possible to detect its presence by activity measurements. When the upper portion of the preparation cen-

trifuged at pH 9.4 on the angle centrifuge was tested, not on the basis of the total protein content, but on the basis of the high molecular weight virus protein content, its activity, as may be seen from the results given for Test 2 in the last section of Table IV, was not found to be significantly different from that of the corresponding lower portion.

As a whole, the experiments with tobacco mosaic protein and with aucuba mosaic protein demonstrate that, as negatively or positively charged protein ions are centrifuged from solution, there is a loss of virus activity and that the decrease in virus activity is proportional to the decrease in the concentration of the high molecular weight protein. This proves that under various conditions the virus and the high molecular weight protein sediment at the same rate and that they are the same size. The results indicate, therefore, that the virus and the protein are identical.

In the experiments described above, centrifugation of solutions containing from about 12 to 20 mg. of virus protein per cc. for 7 to 10 hours on an angle centrifuge at about 5000 R.P.M. resulted in the sedimentation of about 95 per cent of the protein from the upper portion of the supernatant liquid. It seemed of interest to determine how completely the virus protein could be sedimented by means of a short run in an ultracentrifuge. Accordingly, 5 cc. of a solution containing 10 mg. of virus protein per cc. were centrifuged at about 25,000 R.P.M. for about 3 hours. The upper 50 per cent and the next 30 per cent of the clear supernatant liquid were removed separately and tested for protein content and virus activity. The crystalline mass at the bottom of the tube was dissolved in 5 cc. of 0.1 M phosphate buffer at pH 7 and also tested for virus activity. The activity results, which are given in Table V, show that the upper half of the supernatant liquid was less active than the solution of the crystalline protein diluted 100,000 times, and that the next 30 per cent of the supernatant liquid was slightly less active than the protein solution diluted 1000 times. If the activity of these two portions of the supernatant liquid is proportional to the protein present, then, since the original contained 10 mg. of protein per cc., the upper half of the supernatant liquid should contain less than 0.0001 mg. of protein per cc. and the next 30 per cent should contain slightly less than



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0.01 mg. of protein per cc. Since solutions containing less than about 0.01 mg. of protein per cc. fail to give the usual tests for a protein, it appeared likely that, if no more protein were present in the supernatant liquid than the amount proportional to the activity, it would be impossible not only to measure the amount of protein present, but even to detect its presence by the usual chemical tests, particularly in the case of the upper half of the supernatant liquid. This proved to be the case, for the upper half of the supernatant liquid gave negative tests for protein when tested by the usual chemical methods, such as the biuret or Mil-

TABLE V

*Activities of Upper and Middle Portions of Supernatant Liquid and of Sedimented Protein Following Ultracentrifugation of 5 Cc. of Solution Containing 10 Mg. of Crystalline Tobacco Mosaic Virus Protein per Cc. in 0.1 M Phosphate Buffer at pH 7*

All dilutions were made with 0.1 M phosphate buffer at pH 7. The solution of sedimented protein was prepared by dissolving the crystalline mass of protein at the bottom of the centrifuge tube in 5 cc. of 0.1 M phosphate buffer at pH 7.

| Dilution                                 | Undiluted | 1:10  | 1:100 | 1:1000 | 1:10,000 | 1:100,000 |
|--|-----------|-------|-------|--------|----------|-----------|
| Upper 50% of supernatant . . . . .       | 3.4*      | 1.9   | 0.5   | 0.0    | 0.0      | 0.0       |
| Next 30% " " . . . . .                   | 107.2     | 33.5  | 5.8   | 1.5    | 0.1      | 0.0       |
| Solution of sedimented protein . . . . . | 392.0     | 455.5 | 276.5 | 187.0  | 38.7     | 9.4       |

\* The numbers represent the average number of lesions per leaf obtained on ten leaves of *Nicotiana glutinosa* on inoculation with the designated preparation and dilution.

lon's test or coagulation by heat or trichloroacetic acid, and hence contained definitely less than 0.01 mg. of protein per cc. Despite these negative chemical tests, this solution could, of course, contain the 0.0001 mg. of protein per cc. indicated by the activity tests. The next 30 per cent of the supernatant liquid was found to give a very faint cloudiness on addition of an equal volume of hot 5 per cent trichloroacetic acid, and hence probably contained slightly less than 0.01 mg. of protein per cc., which would be in accord with the amount indicated by activity measurements. The results indicate that, when virus protein is sedimented from solution so completely that less than about 0.1 per cent of the

original protein remains, the activity of the supernatant liquid is no greater than that which would be proportional to the amount of protein present.

The writer is greatly indebted to Dr. Ralph W. G. Wyckoff for making available his newly developed air-driven ultracentrifuge (11) and for his generous cooperation throughout the course of the work. It would have been impossible to perform certain of the experiments if the quantity ultracentrifuge had not been available.

#### SUMMARY

Ultracentrifugation of solutions of mixtures of tobacco mosaic virus protein and tobacco proteins, egg albumin, trypsin, and pepsin, respectively, resulted in the sedimentation of the high molecular weight virus protein in the form of a crystalline mass at the bottom of the tube and in the concentration of the virus activity in this protein mass. The supernatant liquids containing the low molecular weight proteins were found to possess practically no virus activity. This indicates that the virus activity is not due to an agent which may be separated from the high molecular weight protein and transferred to other proteins, such as low molecular weight tobacco proteins, egg albumin, trypsin, or pepsin.

When virus proteins were centrifuged at their isoelectric points or when negatively or positively charged virus protein ions were centrifuged from solution on an angle centrifuge so that the upper portion of the supernatant liquids contained from about 5 to 15 per cent of the original protein, the virus activity of different portions of the centrifuged preparations was proportional to the amount of high molecular weight protein present. Ultracentrifugation of over 99.9 per cent of the high molecular weight virus protein from solution yielded a supernatant liquid possessing an activity approximately proportional to the protein remaining in solution. The results demonstrate that in solutions at different hydrogen ion concentrations and in the presence of other proteins the virus activity remains with the high molecular weight protein and are evidence, therefore, that the virus and the protein are identical and hence that the virus activity is a specific property of the high molecular weight protein.

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# A REAGENT FOR THE COPPER-IODOMETRIC DETERMINATION OF VERY SMALL AMOUNTS OF SUGAR

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In sugar determinations based upon the reduction of cupric copper the cuprous oxide formed in the reaction is subject to partial reoxidation unless the operations are carried out in air-free vessels, as in one of Bang's methods. There are several analytical methods, with carefully standardized technique, in which reoxidation does not impair the accuracy of the results. Only in the determination of very small quantities of sugar, a point is reached below which the amount of the cuprous oxide reoxidized is no longer negligible in relation to the total, and slight variations in the extent of reoxidation affect appreciably the accuracy and reproducibility of the results.

Ferricyanide reagents, employed in the Hagedorn-Jensen method (1) and its several modifications, and alkaline mercuric potassium iodide solutions, used in Baudouin's method (2), are free of this drawback, since the reduction products they yield with sugars are not readily reoxidized by atmospheric air. In view of this advantageous quality, we have made an extensive study of the use of these two groups of reagents in the determination of very small quantities of sugar.

As regards ferricyanide solutions, we have reported some years ago, and our finding has been amply confirmed, that the ferric ion is considerably less specific in its reaction with sugars than copper, in that it oxidizes substances other than sugar to a far greater extent than the cupric ion. This fact is known seriously to affect blood sugar values, unless special precipitants are used to remove the non-sugar reducing materials. We find the non-specificity of ferricyanide to be even more disturbing in the analysis of polysaccharides, particularly in cases in which sugar is to be determined

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in extracts of hydrolyzed tissues. Our efforts to eliminate or mitigate this defect remained fruitless.

We have had no more success with mercuric salt solutions. Baudouin's method was found to be grossly inaccurate, mainly due to the fact that sugars begin rapidly to reduce the mercuric ion at room temperatures. The process must be carried to completion by a brief period of heating in a water bath, during which the rate and character of the reaction are altered; thus the reduction equivalents vary according to the relative share of each of the two phases in the reaction. Adequate standardization of the technique being difficult, the results are very inconsistent. We have devised a variety of alkaline mercuric salt solutions in an attempt to eliminate this weak spot of Baudouin's method, but without success.

Thus we turned back to the often approached task of eliminating or at least minimizing reoxidation in copper reagents by some simple step. A reexamination of the influence of electrolytes upon reduction equivalents led to a solution of the problem, which is simple and appears to be satisfactory. It is known that halides diminish, while sulfates increase copper reduction equivalents of sugars. The effect of the halides has been fully explained (3), but the influence of sulfates has, to our knowledge, not been investigated and understood. Our recent studies have shown that sodium sulfate raises the reduction equivalent of sugars in copper reagents, in the main (although not exclusively) by virtue of the fact that the presence of the salt depresses the solubility of oxygen in the reaction mixture. Thus, by the simple addition of 20 per cent of sodium sulfate to a Shaffer-Somogyi reagent, the reoxidation of cuprous oxide in the standard procedure of sugar determination becomes virtually nil, with the result that much smaller amounts of sugars can be determined than with any existing copper-iodometric reagent. For example, while the Shaffer-Somogyi Reagent 50 can still be used for the determination of as little as 0.05 mg. of glucose, consistent results, such as would satisfy a meticulous investigator, are not obtainable with less than 0.15 or 0.20 mg. The addition of 20 per cent sodium sulfate to the reagent, coupled with a diminution of its KI content, so modifies the conditions that as little as 0.01 mg. of glucose can be determined with uniformly consistent and duplicable results.

In adapting Reagent 50 to greater usefulness in microdetermina-

tions, we decreased its KI content to 1.5 gm. per liter. With this KI concentration the Shaffer-Somogyi reagents form some cuprous oxide on standing. We found that the incorporation of sodium sulfate stabilizes the solution and does away with self-reduction, even if the amount of KI is diminished to 1 gm. per liter. It was observed, furthermore, that KI up to 2 gm. per liter does not depress the reduction values obtained with the improved reagent; as a consequence, a reagent which contains 1.5 gm. of KI per liter gives the same reduction equivalents as a KI-free solution.

The sulfate has still another effect upon the reagent: It depresses the ionization of the carbonate, *i.e.* the alkalinity of the solution. This change, as known, augments the reduction equivalents—a welcome influence—but at the same time it also slows down the reaction velocity. A heating period of 20 minutes is, therefore, required to carry the oxidation of glucose sufficiently near completion. Heating for 15 minutes, as will be seen, is acceptable, yet we prefer the 20 minute period for reasons stated in a previous paper (3). Should circumstances arise requiring greater speed, a reagent of the Shaffer-Somogyi series with higher alkalinity can be adapted to microanalysis; thus, by simply replacing the carbonate-bicarbonate buffer mixture with 50 gm. of carbonate per liter of reagent, the heating period is cut to 10 minutes. The higher alkalinity, however, leads to diminished reduction equivalents. In this respect the difference between the two reagents is considerable, in that with the reagent of lower alkalinity 0.50 mg. of glucose reduces an amount of copper equivalent to 4.60 cc. of 0.005 N iodine, while the corresponding figure for the reagent of higher alkalinity is only 3.35 cc. This fact disqualifies the latter for the determination of less than 0.03 mg. of glucose. The composition which we consider most satisfactory for the low alkalinity reagent is as follows:

|   |  |
|---|--|
| 25 gm. $\text{Na}_2\text{CO}_3$ (anhydrous)   | 200 gm. $\text{Na}_2\text{SO}_4$ (anhydrous, |
| 25 " Rochelle salt                            | analytical grade)                            |
| 4 " $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ | 1.5 gm. KI                                   |
| 20 " $\text{NaHCO}_3$                         | 6 cc. N $\text{KIO}_3$                       |

*Preparation*—The carbonate and Rochelle salt are dissolved in about 800 cc. of water; then 40 cc. of a 10 per cent  $\text{CuSO}_4$  solution are introduced with stirring. This is followed by the addition of the bicarbonate, sulfate, and iodide. The solution is heated to

## 774 Reagent for Microdetermination of Sugar

boiling, kept boiling for about 30 seconds, cooled, and after addition of the  $\text{KIO}_3$  diluted to 1 liter.

Since the chemicals, even though of analytical grade, contain solid impurities, the reagent requires filtration. Upon standing for a day or two before filtration, the impurities settle out as flocculent particles and can be removed more completely than if the solution is filtered fresh.

The analytical procedure with this reagent is the same as described in previous papers. In Table I are presented the reduction equivalents of various amounts of glucose obtained after 15 and after 20 minutes of heating.

As may be noted, the useful range of the reagent extends down to 0.01 mg. of glucose (contained in 5 cc. of fluid) and reaches 0.50 mg. as the upper limit. The top concentration is determined by the  $\text{KIO}_3$  content of the reagent (6 cc. of  $\text{N KIO}_3$  per liter), which in turn is contingent upon the amount of KI present. This limitation, however, is not rigid. Should it develop, for instance, that the glucose content of an unknown solution unexpectedly exceeds the 0.50 mg. mark (a practised eye can judge this from the amount of cuprous oxide formed), one can add more iodide and iodate before titration. For this purpose a reagent, which includes both the necessary iodide and iodate, is prepared as follows: in a volumetric flask 2 gm. of KI and 2 gm. of  $\text{Na}_2\text{CO}_3$  are dissolved in 50 cc. of 0.1  $\text{N KIO}_3$  (or  $\text{K(HIO}_3)_2$ ) and the solution is diluted to 1 liter. This reagent, 0.005  $\text{N}$  with respect to the iodate, is quite stable; 5 cc. of it, introduced into the test-tubes before acidifying for titration, yield iodine sufficient to take care of cuprous oxide produced by an extra 0.50 mg. of glucose.

Thus, both accuracy and range in the microdetermination of sugars have been substantially augmented by a practically complete prevention of the reoxidation of cuprous oxide. As a rule this is accomplished by the presence of sodium sulfate in the reagent. But we wish to call attention here to a factor that, if overlooked, may partially frustrate this effect of the salt. It was found, namely, that solid particles, suspended in the reaction mixture, enhance the reoxidation of cuprous oxide. It was an observation made by Benedict that directed attention to this fact. Benedict pointed out (5) that zinc filtrates of blood which contain slight quantities of zinc yield lower sugar values than the same filtrates after removal of the zinc. We can confirm this finding

TABLE I  
Reduction Equivalents of Glucose Expressed in 0.005 N Thiosulfate

| Glucose | 20 min. heating | 15 min. heating |
|---------|-----------------|-----------------|
| mg.     | cc.             | cc.             |
| 0.5     | 4.60            | 4.40            |
| 0.2     | 1.84            | 1.72            |
| 0.1     | 0.91            | 0.87            |
| 0.05    | 0.49*           | 0.47            |
| 0.02    | 0.21            | 0.20            |
| 0.01    | 0.13            | 0.11            |

\* As may be noted, the reduction equivalents are in linear proportion with the amounts of glucose, evidently due to the prevention of reoxidation. This proportionality, however, only prevails when the amount of glucose involved is more than 0.05 mg., while with 0.05 mg. and less sugar the reduction equivalent rises, and the increase becomes gradually greater as the amount of the sugar diminishes. Thus, with 0.05 mg. of glucose the rise is about 6 per cent, with 0.02 mg. it is 14, and with 0.01 mg. it leaps to 40 per cent. (In the last instance the titration value is 0.13 cc. of 0.005 N thiosulfate instead of 0.09 cc., as computed from the value obtained with 0.1 mg. of glucose.) This phenomenon contradicts all experiences with existing copper reagents, but can be readily understood. We have pointed out in a previous paper (4) that the amount of copper reduced by glucose is the resultant of two competing processes. On the one hand, fragments of the glucose molecule, which could be oxidized, tend to escape oxidation by entering into addition products; on the other hand, there is the oxidative process itself, tending to intercept the oxidizable units. Increase in alkalinity, as we have said (4), enhances the rate of the first process and, as a consequence, lowers reduction equivalents. In the present instance it is the rate of oxidation as a function of the relative concentration of the cupric ion that is responsible for the shift in the reduction equivalents. Of necessity the copper must be always in excess of the amount required to oxidize the sugar, yet this excess may vary considerably without any measurable effect upon reduction equivalents. When, however, the concentration of the copper is *overwhelmingly high* in relation to that of the sugar (as in our reagent when the amount of glucose is 0.05 mg. or less per 5 cc. of solution), then the rate of oxidation outstrips that of the competitive reaction sufficiently to cause substantial augmentation of the reduction equivalent. The validity of this explanation is borne out by the fact that the reduction equivalent can also be increased for 0.1 mg. and more of glucose, merely by a commensurate increase of the copper content of the reagent.

and add that the larger the amount of zinc present the greater is the depression of the sugar values. The explanation is this: Zinc salts contained in the filtrate form with copper reagents a fine



flocculent precipitate upon which, in the course of the reaction, cuprous oxide settles out in an extremely fine state of dispersion; convection currents during the heating period move the precipitate time and again to the surface, exposing the cuprous oxide to the access of air and thereby to reoxidation. This accounts for the depression of the reduction values. This interpretation is verified by the fact that with ferricyanide reagents the presence of zinc precipitates has no effect upon the reduction values, obviously because reoxidation of ferrocyanide does not occur.

This source of error is not confined to zinc filtrates of blood. It occurs, for example, in a frequently employed procedure in which biological material is prepared for sugar determination by removing nitrogenous matter with mercuric salts. In the process some mercury is always held in solution and must be eliminated by treatment with either  $H_2S$  or powdered zinc. In the latter case zinc passes into solution, frequently in considerable amounts, and may gravely affect the results, especially when the amount of sugar under determination is very small. It should be observed, therefore, as a general rule, that suspended solid particles and substances forming precipitates with copper reagents must be removed from sugar solutions before the analysis is executed.

#### SUMMARY

A reagent for the copper-iodometric determination of sugars is described, in which the reoxidation of cuprous oxide by air is virtually eliminated. As a consequence, first, very small quantities of sugars (as little as 0.01 mg. of glucose) can be determined accurately by the copper-iodometric technique, and second, a linear proportionality between the amounts of the sugars and the copper reduced is established. Thus, the advantages of ferricyanide reagents have been imparted to copper reagents, without the tendency of the former to oxidize extensively substances other than sugar.

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## CONTRIBUTIONS TO THE STUDY OF MARINE PRODUCTS

### IV. THE STEROLS OF STARFISH

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That the common starfish *Asterias rubens* contains a sterol different from cholesterol was first reported by Dorée in 1909 (1). His observation was corroborated by Kossel and Edlbacher in 1915 (2) who found that in the tissues of the starfish *Astropecten aurantiacus* cholesterol is replaced by a substance of the formula  $C_{27}H_{44}O$  which was named stellasterol. This new sterol, which melted at 149–150°, gave an acetate, m.p. 176–177°, and a benzoate, m.p. 100–125°. Besides stellasterol Kossel and Edlbacher isolated from the non-saponifiable material of the starfish astrol, an alcohol of the formula  $C_{28}H_{48}O_3$  and with a melting point of 71°.

Quite different results were obtained by Page (3). This investigator isolated a sterol-like substance from *Asterias forbesi*, the common starfish of the coast of New England, which he named asteriasterol. Asteriasterol melted at 71°, a melting point which is a surprisingly low one for a naturally occurring sterol. For further identification an acetate with a melting point of 97° and a benzoate melting at 125° had been prepared. No details concerning the purity of the isolated substances, however, have been given, nor have analyses of the sterol or its derivatives been reported.

That asteriasterol is a pure substance seemed rather doubtful. Its low melting point and especially the observation that it is only incompletely precipitated by digitonin suggest that it is a mixture of Kossel's astrol and stellasterol. In order to determine whether that is the case, the author has reinvestigated the non-saponifiable material of *Asterias forbesi*.

When the ether extract of dried and ground starfish is saponified

with alcoholic potassium hydroxide, a non-saponifiable material is obtained from which by recrystallization with alcohol a substance can be obtained which has properties similar to those of asteriasterol. It shows a low melting point of 70–80° and the usual color reactions of unsaturated sterols.

When treated with digitonin in an alcoholic solution, only about 60 per cent of the material could be obtained in form of an insoluble digitonide. The sterol which was recovered from the digitonide, with the help of Schoenheimer's method, showed a melting point of 130–140°. From the mother liquor of the digitonide a substance could be extracted with ether, which did not show the usual sterol reactions and which closely resembled astrol. Asteriasterol therefore must be considered a mixture of one or more sterols and a substance like astrol.

By using the method which has been employed by Kossel, a preliminary separation of the sterols and astrol can be effected. While astrol itself is rather soluble in alcohol, it is present in the starfish in the form of an ester which is comparatively insoluble in boiling alcohol. Consequently by first preparing an alcoholic extract of the starfish and then saponifying the ether-soluble part of this extract, a sterol can be obtained which is comparatively free from astrol.

For the preparation of sterol the starfish were first dried rapidly *in vacuo* at 50° and then ground to a fine powder. Batches of 100 gm. of this powder were then extracted once with 500 cc. and twice with 250 cc. of boiling alcohol. The extracts were combined and evaporated to dryness. The residue, which contained considerable amounts of free amino acids, was then extracted with ether. From the ether extract 3.5 gm. of a dark brown oil were obtained which after saponification with alcoholic potassium hydroxide rendered 1.2 gm. of an orange-colored non-saponifiable material. After two recrystallizations from methanol 0.8 gm. of a crystalline substance was obtained of which from 80 to 90 per cent could be precipitated with digitonin. The sterol which was recovered from the digitonide melted between 135–145°. It gave the same color reactions as Kossel's stellasterol. Of these, the reaction with bromine, which leads to a green coloring, is especially characteristic. The substance shows  $[\alpha]_D^{20} = +2.7^\circ$  and no absorption in the ultraviolet region between 230 and 350  $m\mu$ . Further re-

crystallizations demonstrated that one had to deal with a mixture of at least two different sterols. After twelve recrystallizations from alcohol the less soluble sterol melted at 149–150°. It was then distilled at 250° and 0.01 mm. pressure and the distillate recrystallized twice from alcohol and ethyl acetate. The substance now melted at 154–155°. During all recrystallizations the specific rotation underwent very little change. The final product showed  $[\alpha]_D^{20} = +3.0^\circ$ .

*Rotation*—0.121 gm. of substance dissolved in  $\text{CHCl}_3$  and made up to 3 cc. gave in a 1 dm. tube a reading of  $+0.12^\circ$ ; hence  $[\alpha]_D^{20} = +3.0^\circ$ .

The sterol closely resembled stellasterol. Its analysis supports the formula  $\text{C}_{27}\text{H}_{44}\text{O}$  which has been proposed for stellasterol.

*Analysis*—4.671 mg., 14.365 mg.  $\text{CO}_2$ , and 4.8 mg.  $\text{H}_2\text{O}$   
 $\text{C}_{27}\text{H}_{44}\text{O}$ . Calculated. C 84.30, H 11.54  
Found. “ 83.90, “ 11.64

The acetate of the sterol after six recrystallizations from alcohol melted at 155–157°,  $[\alpha]_D^{20} = +7.0^\circ$ .

The melting point of the acetate obtained differs greatly from that of stellasterol acetate which after twelve recrystallizations was found to be 176–177°.

*Analysis*—4.072 mg., 12.173 mg.  $\text{CO}_2$ , and 3.992 mg.  $\text{H}_2\text{O}$   
 $\text{C}_{27}\text{H}_{44}\text{O}_2$ . Calculated. C 81.62, H 10.87  
Found. “ 81.55, “ 10.97

*Titration with Perbenzoic Acid*—The acetate was titrated with perbenzoic acid in the usual manner. After 24 hours 44 mg. of acetate had used 3.2 mg. of oxygen, corresponding to 1.9 double bonds.

*Benzoate*—The benzoate was prepared in the usual manner by treating the sterol in dry benzene with an excess of benzoyl chloride. It was recrystallized five times from a mixture of alcohol and ether. The benzoate crystallizes in fine needles. When heated in a capillary tube, it melts at 177° to a turbid liquid, which after a play of colors becomes clear and colorless at 182°. This melting point differs greatly from that of a stellasterol benzoate which has been found to melt at 100° and to become clear at 125°.

*Analysis*—4.831 mg., 14.775 mg. CO<sub>2</sub>, and 4.44 mg. H<sub>2</sub>O  
 C<sub>34</sub>H<sub>48</sub>O<sub>3</sub>. Calculated. C 83.45, H 9.90  
 Found. " 83.45, " 10.29

*3,5-Dinitrobenzoate*—The 3,5-dinitrobenzoate was prepared by heating equal amounts of sterol and 3,5-dinitrobenzoyl chloride in 10 times the amount of dry pyridine on the steam bath for 1 hour. After cooling, the mixture was poured into dilute sulfuric acid, and the precipitate was filtered, washed with water and acetone, and recrystallized a few times from ethyl acetate. The 3,5-dinitrobenzoate crystallized in the form of slender, slightly yellow needles, m.p. 194–195°.

*Analysis*—4.196 mg., 10.808 mg. CO<sub>2</sub>, and 3.075 mg. H<sub>2</sub>O  
 C<sub>34</sub>H<sub>46</sub>O<sub>6</sub>N<sub>2</sub>. Calculated. C 70.54, H 8.02  
 Found. " 70.27, " 8.20

From the mother liquors of the less soluble sterol a more soluble substance with a melting point of 128–130°,  $[\alpha]_D^{20} = +2.4^\circ$ , was obtained. It gave an acetate and a benzoate melting at 128–130° and 130–135° respectively. Attempts to purify these substances further by a series of recrystallizations failed because of lack of material. Several analyses of the sterol and its derivatives gave no agreement. They all showed a carbon content which was from 2 to 3 per cent below that required for a substance of the formula C<sub>27</sub>H<sub>44</sub>O.

The preliminary results presented in this investigation demonstrate that the starfish *Asterias forbesi* contains a complex mixture of at least two different sterols, which are very difficult to separate. Unfortunately the various fractions obtained during recrystallization show such little variation of their specific rotation that it is of no avail as an indicator of purity. In order to obtain pure substances it will be necessary to carry out a process of separation with considerably more material than was used before. Until such separation has been carried out it does not seem advisable to name the various fractions of sterols from starfish.

#### SUMMARY

It has been shown that asteriasterol, the sterol which had been isolated from *Asterias forbesi* is not a uniform substance, but rather a mixture of an alcohol resembling astrol and two or more sterols.

The least soluble of these sterols shows in its properties and composition some resemblance to stellasterol. The acetates and benzoates, however, showed considerable differences in their respective melting points. From the experimental work presented it seems very doubtful that stellasterol is a uniform substance. A more soluble sterol with a melting point of 128–130° could only be obtained in a low degree of purity. Further investigations of larger amounts of material will be necessary before any one of these sterols can be definitely identified.

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## STANLEY ROSSITER BENEDICT

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With profound regret the Journal records the death on December 21, 1936, of Stanley R. Benedict. His contributions to the progress of biological chemistry entitle him to high rank. A fruitful investigator of many topics in the field of metabolism, he designed numerous analytical methods which found wide and important uses both in clinic and in research. During thirty years he contributed more than seventy papers to this Journal. A member of its Editorial Board for seventeen years, he was for the last eleven the Managing Editor. Few can realize the magnitude of this editorial service, or the personal sacrifice it demanded of him. No one could have given more generously of himself to the development of biochemical science; no one has served both contributors and readers of this Journal more devotedly, or more efficiently. As a teacher at Cornell University Medical College, for twenty-five years he stimulated colleagues, aroused the interest of students in the chemical aspects of physiology and medicine, encouraged honest ability, and fought sham and hypocrisy when it came his way. A staunch loyalty to ideals and to facts as he saw them, a scorn for pretense, a willingness to strike vigorously in defense of his work and ideas, a warm and generous heart inside a gruff exterior, these are qualities that characterize the man.

Benedict was born at Cincinnati, Ohio, March 17, 1884 and died of heart disease at the age of 52 at his home in Elmsford, New York. He is buried in Forest Hills Cemetery, near Boston. He is survived by his wife Ruth Fulton Benedict, a well known ethnologist whom he married in 1913, and by three sisters.

The influences which gave direction to Benedict's intellectual and scientific interests are to be seen in his inheritance and in his early experiences. His father, Wayland Richardson Benedict, was professor of philosophy at the University of Cincinnati; his mother's father, A. C. Kendrick, was professor of Greek at the University of Rochester. While a college student at the



University of Cincinnati, he came under the stimulus of Dr. J. F. Snell, who had shortly before worked with Atwater. The vigor of that association is indicated by the fact that Benedict published no less than nine papers (on inorganic analytical methods, three jointly with Dr. Snell) before his graduation; the first appeared during his second year in college when he was nineteen years old. Benedict's interest in analytical problems and his remarkable skill and resourcefulness in devising methods are evidenced by his work at this early period; it was then that he formulated his ideas for the sugar reagents which later brought him widest fame.

After the first year in college Benedict decided to enter medicine and spent the next year as a student in the medical school, where also he assisted with the course in chemistry. He soon felt that for him the study of medicine was a mistake and returned to chemistry; but he had demonstrated his ability as a teacher of chemistry and while a college student continued to assist in that course in the medical school.

After graduation in 1906 at Cincinnati he went to Yale to study under Chittenden and Mendel; from them he absorbed his interest in metabolism and a knowledge of physiology. A friend and fellow student at Yale, Professor V. C. Myers, relates that Benedict was then as later quiet, retiring, and studious, deeply interested in philosophy as well as physiology and chemistry, but enjoyed the conviviality of a club of graduate students that met regularly on Saturday nights.

After receiving the degree of Ph.D. at Yale in 1908, one year was spent as instructor at Syracuse University, and another at Columbia. In 1910 Benedict moved to Cornell University Medical College, where, after two years in chemical pathology, he was made professor of chemistry, a position he held with distinction until his death. Lusk was his colleague and friend.

Benedict's career almost parallels and in some respects resembles that of Folin. Both excelled in designing very clever analytical methods of the widest usefulness, and in using these tools with rare success for the discovery of new facts about metabolism. In spite of seventeen years difference in age, of the rivalry and controversy sometimes evident in their papers, there early developed between them a warm friendship which reveals the fine character of both. They were kindred spirits.

An analysis here of Benedict's numerous contributions is unnecessary; they are mostly to be found in the pages of this Journal.

But it may be fitting to record here the fact that Benedict became an editor of the Journal at the time its conduct was entrusted to the American Society of Biological Chemists, an omen which should strengthen the fortunate relation between them. After serving three years as Secretary of the Society, Benedict was elected its President in 1919. During his second term the publication of the Journal was transferred (in 1920 with volume 42) to the Society and Benedict was wisely made a member of the Editorial Board. Six years later Benedict reluctantly accepted the onerous and all-too-thankless task of Managing Editor, and extended to the Journal the hospitality of Cornell University Medical College. From that day until his death Benedict made this Journal his constant concern. With many another contributor the present writer has occasionally smarted under the sometimes sharp criticisms of the editor; but rarely if ever were the criticisms unjustified. His standards were high, he expected clarity, logic, and brevity in exposition, his opinions were definite and outspoken, his judgments were based on essential facts and were impartial as to individuals.

Besides the work of his laboratory and his editorial duties Benedict had little time for other interests. Relaxation he found in the country, in photography, and in philosophical reading. His intimate friends were few; to them he cannot be replaced. His position among biochemists and in the conduct of this Journal will be hard to fill.

PHILIP A. SHAFFER



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